REMARKS

Receipt of the Office Action mailed April 29, 2008 is hereby acknowledged. With the attached Petition for a Three-Month Extension of Time, this response is timely. Reconsideration of the rejections in view of the concurrently filed Request for Continued Examination and allowance of this application, as amended, is respectfully requested.

Amendments

Claim 21, the only independent claim currently under examination, has been amended to exclude the presence of vitamin K from the composition used in the claimed method. This amendment is supported by the recitation of vitamin K in the specification at page 16, line 28 as a possible component of the composition used in the claimed method. (*See, In re Johnson*, 558 F.2d 1008, 1019, 194 USPQ 187, 196 (CCPA 1977) (a negative limitation excluding an element is properly supported by a recitation of that element in the specification.)

Claims 4, 5, 7, 15, 16, and 18 have been amended to replace the word "prevent" as previously used in the claims, with the phrase: "reduce an imbalance in bone remodelling." This is supported in the specification at page 2, lines 28-30, where "preventative treatment" of bone loss is described as a reduction in bone remodelling. Claim 8, 9, and 19 have also been amended to employ more conventional English grammar. No substantive change has been made and no new matter has been added.

No new matter has been added, and entry of the foregoing amendments are respectfully requested.

Rejections Under 35 U.S.C. § 112

Claims 4, 5, 7, 15, 16, and 18 have been rejected under 35 U.S.C. § 112, first paragraph as allegedly not enabled. According to the Examiner, these claims are not enabled with respect to the issue of "preventing" the various indications specified in the claims. Applicants respectfully traverse. The claims have been amended to recite that the claimed method reduces an imbalance in bone remodeling, which is exemplified, as discussed in applicants' previous amendment, in Examples 1 and 3. The Examiner's apparent concern with the showings of Examples 1 and 3, as well as the disclosures of Shen, et al., Bone, Vol. 20 (No. 1): 55-61 (1997) ("Shen") (a copy of which was submitted with the last amendment) was that the preventative effect they demonstrated might not be "total, absolute, or permanent." While applicants do not agree that the word "prevention" requires a "total, absolute, or permanent" effect, they submit that the currently worded claims are fully enabled within the meaning of § 112. Therefore, reconsideration and withdrawal of the enablement rejection is respectfully requested.

Rejections under 35 U.S.C. § 102(b)

Wenzel

The Examiner has rejected claims 2-9, 11, 12-20, and 22 under 35 U.S.C. § 102(b) as being anticipated by Wenzel, et al., EP 1127572A2 ("Wenzel"). According to the Examiner, Wenzel teaches that "compositions of flavone-type compounds of

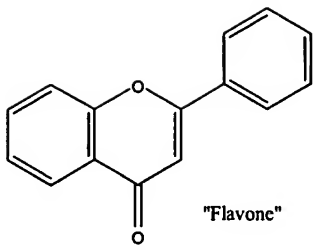
formula I, specifically hesperidin and hesperitin (Table 3) are useful in the treatment of [COX-2] and [NFχB] mediated diseases." Furthermore, according to the Examiner, who cites two different journal articles, COX-2- (Katori, et al.) and NFχB- (Hofbauer) mediated diseases include post-menopausal osteoporosis and other diseases. Based on this, the Examiner concludes: "the limitations of claim 21 are met."[1] Applicants traverse this rejection.

Wenzel relates to the use of flavones for treating COX-2 and NFχB, particularly for treating arthritis and Alzheimer's disease (see ¶0001). Wenzel prescribes the use of flavone compounds for inhibiting the biosynthesis of COX-2 and of NFχB, i.e. as inhibitors of the prostaglandin synthesis. (¶¶ 0001-0005). Numerous compounds that can be used, according to Wenzel, as COX-2 and NFχB inhibitors are shown in Tables 1 to 4 (see pages 3 to 5). According to Wenzel, the diseases linked to COX-2 consist of diseases linked to inflammation, mitogenesis, and ovulation (¶ 0002). Paragraph 0023 specifies a general and theoretical list of diseases which might "potentially" be treated by Wenzel's compounds, including osteoporosis among 16 categories of diseases.

The only experimental results disclosed by Wenzel are disclosed in Example 3, which shows that a specific flavone compound induces an inhibition of the expression level of the messenger RNA's corresponding to the transcription product of the genes encoding COX-2 and NFχB. Thus, Wenzel discloses experimental results relating to the inhibition of the expression of the genes and coding for COX-2 and NFχB by this flavone:

□

[1] Claim 21 is not included in the original formulation of the rejection set forth on page 7 of the Final Rejection. It is assumed that this is simply a typographical error.



"Flavone"

(See structure of "flavone" as identified in Table 1 on page 3). The inhibitory effect of this compound on COX-2 and NFχB, the mRNA levels is shown for an *in vitro* final concentration of 150 μM. Wenzel's exemplified compound – "flavone"- does not belong to the group of compounds used in applicants' claimed method.

As is well known to the Examiner, an allegedly anticipating reference needs to "describe the applicant's claimed invention sufficiently to have placed it in possession of a person of ordinary skill in the field of the invention." *Arthrocare Corp. v. Smith & Nephew, Inc.*, 406 F.3d 1365 (Fed.. Cir. 2005). Moreover, the allegedly anticipating reference must enable that which it is asserted to anticipate. *Elan Pharmaceuticals, Inc. v. Mayo Foundation for Medical Education and Research*, 346 F.3d 1051 (Fed. Cir. 2003). The reference must allow one of ordinary skill in the art to make the invention without undue experimentation. *Id.*

The presently pending claims in the present application are directed to a method for stimulating bone formation and/or inhibiting bone resorption by administration of hesperidin and other derivatives. Wenzel appears to enable the administration of "flavone" to inhibit COX-2 and NFχB mRNA expression. That it may be "well known in the art that inhibition of COX-2 and NFχB activity can be employed in the treatment of

osteoporosis and other bone related diseases" (Final Rejection, p. 3)[2] does not enable the use of any or all of the compounds disclosed in Wenzel to stimulate bone formation and/or inhibit bone resorption without undue experimentation. In particular, one of skill in the art would have had to select hesperidin from among the thousands of compounds disclosed by Wenzel. He or she would then have to select hesperidin to treat osteoporosis, or another bone related disease, and determine that administration of the compound would treat the disease. The Examiner has not pointed to anything showing a direct link between the inhibition of COX-2 or NFχB activity and the effect covered by the presently claimed method. Moreover, as discussed in some detail below, a person of skill in the art would have ample reason to be dubious about the transferability of the demonstrated results from Wenzel (using "flavone") to the compounds used by the presently claimed method. Accordingly, Wenzel does not anticipate the present claims.

The single compound exemplified by Wenzel, which it dubs "flavone," is an aglycone polyphenol, while the compounds used in the presently claimed method consist of glycosylated or sulphated polyphenols. These two types of compounds behave differently *in vitro* and *in vivo*.

□

[2] Applicants do not agree with the Examiner's position here. Katori is a review article on the possible functional roles of COX-2. Notably, Katori indicates that COX-2 induction or presence has been reported in a wide variety of physiological states, including "bone absorption." At most, one of skill in the art learns that the presence of COX-2 is noted in states of bone absorption. This information does nothing to convey to one skilled in the art the role of COX-2 in bone remodeling, much less whether its presence, inhibition, or stimulation would have a specific effect on bone remodeling.

Hofbauer describes the role of a receptor activator of NFχB (RANKL) and of NFχB. Hofbauer are interested in the physiological relationship of RANKL with another receptor (OPG). With respect to bone diseases, Hofbauer merely discloses that "Abnormalities of the RANKL/OPG system have been implicated in a wide variety of diseases, including postmenopausal osteoporosis. Again, it is not made clear whether stimulation or inhibition would have a particular effect on bone diseases.

As shown in the attached article from Serra, et al., "*Prediction of intestinal absorption and metabolism of pharmacologically active flavones and flavanones*," Bioorganic and Medicinal Chemistry, **16** (2008) 4009-4018 ("Serra", attached hereto as Exhibit A), *in vitro* cell permeation is not detected for the glycosides. Serra further shows that the glycosylated flavonoids, when administered *in vivo*, need to be metabolized before being absorbed through the intestinal membrane. (*See* Serra, Abstract). Serra states that: "*no transport across the cell layer or metabolism was detected with the glycosylated flavonoids, neither in the presence nor the absence of glucose.*" (Serra, p. 4012). Consistent with this, Serra concluded that "*hesperidin, diosmin and narangin did not permeate, confirming other author's observations of low permeabilities for flavonoids glycosides.*" (Serra, p. 4014). By contrast, Serra noted that "*the aglycones permeated across the membrane to the acceptor compartments in both the apical to basolateral and basolateral to apical assays.*" (Serra, p. 4012).

In summary, Serra clearly demonstrates that the non-glycosylated polyphenols like Wenzel's flavone compound and the glycosylated polyphenols like hesperidin have completely different behaviors, particularly in *in vitro* assays.

Moreover, as shown by Kroon, et al., "*How should we assess the effects of exposure to dietary polyphenols in vitro*," Am J Clin Nutr, **50**, 15-21 (2004) ("Kroon," attached hereto as Exhibit B), *in vitro* assays of alleged effects of polyphenols are physiologically meaningless, particularly regarding aglycone polyphenols, since plasma and tissues are not exposed *in vivo* to polyphenol in these forms. (See, Kroon, Abstract). Kroon also emphasized that polyphenol concentrations in plasma are at maximum range of 0.1 to 10 μmol/L, and thus *in vitro* assays of polyphenol effects should respect this

final concentration. *Id.* The results on COX-2 and NFχB mRNA levels obtained by Wenzel were obtained with a final *in vitro* concentration of 150 μM (see Table 5 on page 7 of Wenzel). Thus, whatever teachings Wenzel might provide would be known to by physiologically meaningless. This further illustrates why Wenzel cannot be said to be enabled with respect to the presently claimed invention.

Because of the different known behaviors of the various classes of compounds identified in Wenzel, as well as the difficulty in translating Wenzel's effect of "flavone" on COX-2 and NFχB mRNA levels into the effects of hesperidin on bone loss, Wenzel cannot be said to be enabled with respect the use of hesperidin for the treatment of diseases characterized by bone loss. Consequently, applicants respectfully submit that the anticipation rejection of claim 21 should be reconsidered and withdrawn, as well as the anticipation rejections of the remaining claims over Wenzel.

Kise, et al.

Claims 2-7, 9-10, and 13-19, and 21-22 have been rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Kise, et al., JP 2001114675A ("Kise"). The Examiner claims that Kise teaches a vitamin composition containing vitamin K and flavonoids, including hesperidin, and that Kise further teaches that "compositions of vitamin K, vitamin D3, estrogen, isoflavone, etc. are known to prevent and treat osteoporosis." Applicants respectfully traverse this rejection. Claim 21 now explicitly excludes the presence of vitamin K. Accordingly, Kise, which is directed to the use of vitamin K in combination with a soybean hypcotyls extract and various other

compounds for the treatment of osteoporosis cannot anticipate any of the pending claims. This rejection should be reconsidered and withdrawn.

Rejection Under 35 U.S.C. § 103(a)

The Examiner has rejected claim 9 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Wenzel in view of Barnes, et al., U.S. Patent No. 5,506,211 ("Barnes"). Applicants traverse this rejection.

First, as discussed in detail above, Wenzel does not anticipate the independent claim from which claim 9 depends (claim 21, through claim 2). Nothing in Barnes remedies the deficiencies of Wenzel in this regard (enablement). Simply put, one of ordinary skill in the art would have known, notably from the disclosures of Serra and Kroon, that the *in vitro* assays of Wenzel would not have been credible for assessing the potential *in vivo* effect of polyphenol on the COX-2 and NFχB expression levels (much less the effect of hesperidin on bone loss). This is because non-glycosylated flavonoids like Wenzel's "flavone" is not a pertinent molecule for *in vitro* assays.[3]

The same remarks may be made regarding the teachings of Barnes. Barnes discloses the use of a specific isoflavone – genistein, which is listed in Table 4 on page 5 of Wenzel – in the treatment of osteoporosis. However, genistein is a completely different compound from hesperidin. Genistein falls within the class of isoflavones while hesperidin is a flavone.

Essentially, like "flavone" as tested by Wenzel, genistein consists of a non-

[3] It is worth noting again that the *in vitro* final concentration of 150 μM used by Wenzel are very high relative to conventional polyphenol plasma concentrations, which are usually at most 10 μM (0 to 4 μM according to Manach, et al. (attached as Exhibit C) and 0.1 to 10 μM according to Kroon.

glycosylated polyphenol, whose effect on osteoclasts has been exclusively exemplified by Barnes with *in vitro* assays (see Barnes, Examples I, II, III, and IV). Example V of Barnes, which refers to "*the use of genistein to stimulate bone resorption in vivo*" actually relates to previous works and technical indications of how *in vivo* administration of genistein might be performed. It does not provide any data that would show that an *in vivo* administration of genistein for inhibiting osteoclasts would be more than incentive to test the actual physiological effect of genistein.

In contrast to the limited relevant technical content of the prior art relied upon by the Examiner – especially Wenzel and Barnes, which disclose only *in vitro* administration using non-physiological concentration of the alleged active ingredient – the present application exemplifies the in vivo effect of hesperidin on bone remodelling, and not only:

- the effect of "flavone" on gene expression of genes involved in inflammatory reactions like COX-2 and NFχB (like Wenzel), or
- the *in vitro* effect of an isoflavone like genistein on the in vitro metabolism of osteoclasts.

In view of all of the foregoing, applicants submit that claim 9 would not have been obvious over Wenzel in view of Barnes, and request withdrawal of the rejection.

Conclusion

In view of the foregoing, this application is now in condition for allowance. If the examiner believes that an interview might expedite prosecution, the examiner is invited to contact the undersigned.

Respectfully submitted,

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Exhibit A

Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry
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Bioorganic & Medicinal Chemistry 16 (2008) 4009–4018

Prediction of intestinal absorption and metabolism of pharmacologically active flavones and flavanones

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Received 12 September 2007; revised 8 January 2008; accepted 16 January 2008
Available online 19 January 2008

Abstract—Three glycosilated flavonoids (diosmin, hesperidin and naringin) and respective aglycones were characterized in terms of their apparent ionisation constants and bidirectional permeability using the cellular model Caco-2 as well as the artificial membrane model PAMPA. Ionisation curves were established by capillary electrophoresis. It was confirmed that significant amounts of the aglycones are ionised at physiological pH whereas the glycosides are in the neutral form. Permeation was not detected for the glycosides in either the apical to basolateral or basolateral to apical directions confirming the need for metabolism before absorption through the intestinal membrane. The aglycones permeated in both directions with apparent permeabilities (P_{app}) in the range of $1–8 \times 10^{-5}$ cm/s. The results from both in vitro methods correlated providing some evidence of passive transport however the hypothesis of active transport can not be excluded particularly in the case of diosmetin. Metabolism of the aglycones was detected with the cell model, more extensively when loading in the apical side. Some of the metabolites were identified as glucuronide conjugates by enzymatic hydrolysis.

1. Introduction

Flavonoids are a large group of polyphenolic compounds naturally occurring in several plants and fruits as glycosides or, less frequently, as their aglycones. They are abundant in human diet[1–4] and their role in the prevention of cancer and cardiovascular diseases has attracted substantial attention.[5–7]

Diosmin and hesperidin (Fig. 1) are respectively a flavone and a flavanone rutinoside that can be found mainly in citrus and, in the case of diosmin, in Hyssop and Rosemary.[8–11] The effects of these compounds on the improvement of muscular tone and vascular resistance to inflammatory processes, anti-oxidant activity and ability to quench oxygen free radicals involved in cancer are frequently mentioned.[8–18] In Europe, they are available under medical prescription for the treatment of illnesses such as chronic venous insufficiency[15,19] but despite the current claims of biological activity, there is no general recognition of these compounds as valuable medicines (in the USA, for example, they are commercialised only as food supplements).

Naringin (Fig. 1), a flavanone neohesperidoside mainly found in grapefruit, also exhibits different pharmacological properties such as anti-inflammatory, anti-oxidant, anti-microbial, anti-mutagenic, anti-carcinogenic, cholesterol lowering, and free radical scavenging.[20–23]

Solubility of these flavonoid diglycosides in aqueous solution is low, particularly in the case of diosmin, unless very high pH is used,[24] even in the presence of dissolution aids.

In addition, bioavailability is usually low[25] and permeation is thought to occur only after intestinal metabolism since the diglycosylated forms are not found in the systemic circulation. It is generally recognized that flavonoid diglycosides like rutinosides and neohesperidosides, pass intact through the small intestine.[26] In contrast to glucosides, which can be hydrolysed by glucosidases available throughout the intestinal tract, these diglycosides are hydrolyzed only by rahmnosidases

Keywords: Flavonoids; Caco-2 model; PAMPA; Intestinal absorption; Permeability; Ionisation constants.
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doi:10.1016/j.bmc.2008.01.028

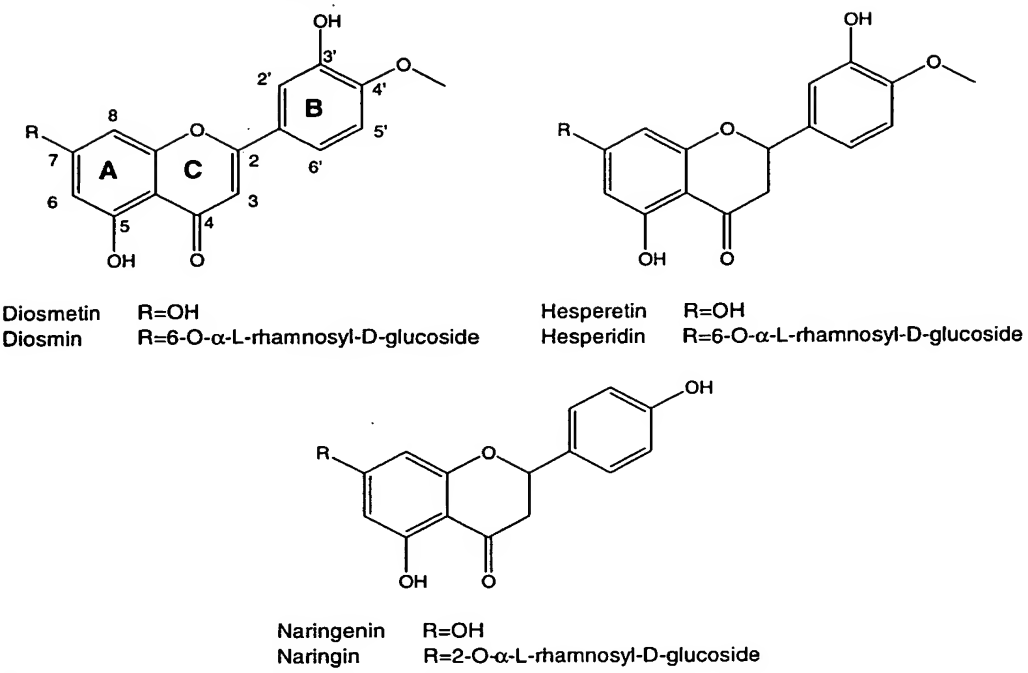

Figure 1. Structures of the flavonoids diosmin, hesperidin, naringin, and their aglycones.

produced by enterobacteria as they enter the cecum and colon. They are thought to be absorbed there as aglycones[27] which are found in the blood as conjugated metabolites such as glucuronides and sulfates.[28–31]

This led us to investigate pre- and post-deglycosilatyon membrane transport of these flavonoids using in vitro models. These models are becoming more popular for prediction of drug bioavailability[32,33] because they lend themselves to automation and high throughput screening, but also because they diminish the need to use animals in preliminary tests where many compounds are discarded. Moreover, being less complex systems they are easier to interpret and correlate with in vivo observations.

Among the in vitro systems used for the study of drug absorption, there are two models which find particular applicability: Caco-2[34] and PAMPA[35] (parallel artificial membrane permeability assay). In the Caco-2 model, permeability is tested across a differentiated monolayer of cells of the human colon adenocarcinoma, while in the PAMPA method an artificial phospholipidic membrane is used for the same purpose. Although cellular models provide more information in terms of active transport, efflux and metabolism, they are more time consuming than artificial membrane models such as PAMPA, which however provide only correlation to passive transport. Combining data from both models may nevertheless provide insights into the mechanism of absorption.

The main objective of the present study was the evaluation of the intestinal epithelial transport and the metabolism of diosmin, hesperidin, naringin and their respective aglycones, as a contribution to understanding the mechanisms responsible for the therapeutic effects of some flavonoid rutinosides.

Permeabilities were determined using the Caco-2 cell model and the artificial membrane model PAMPA. The observations were discussed taking into account other factors that can affect the rate of membrane penetration, like lipophilicity, degree of ionisation and molecular size.

The ionisation characteristics of the compounds were also determined since they have direct implications for membrane permeability. Despite being well known compounds, there is no published data relative to the ionisation constants (pK_a) of the test compounds except in the case of naringin and naringenin.[36,37] There are several methods for pK_a determination but due to its simplicity and selectivity, capillary electrophoresis (CE) has been frequently used[38–43] and was chosen for this purpose in the present work.

2. Results and discussion

2.1. Determination of the ionization profiles

The ionisation curves obtained by non-linear regression fitting of a sigmoidal curve to the effective mobilities of the test compounds against pH are shown in Figure 2. The aglycones possessed lower apparent ionisation constants than the corresponding glycosilated flavonoids (Table 1).

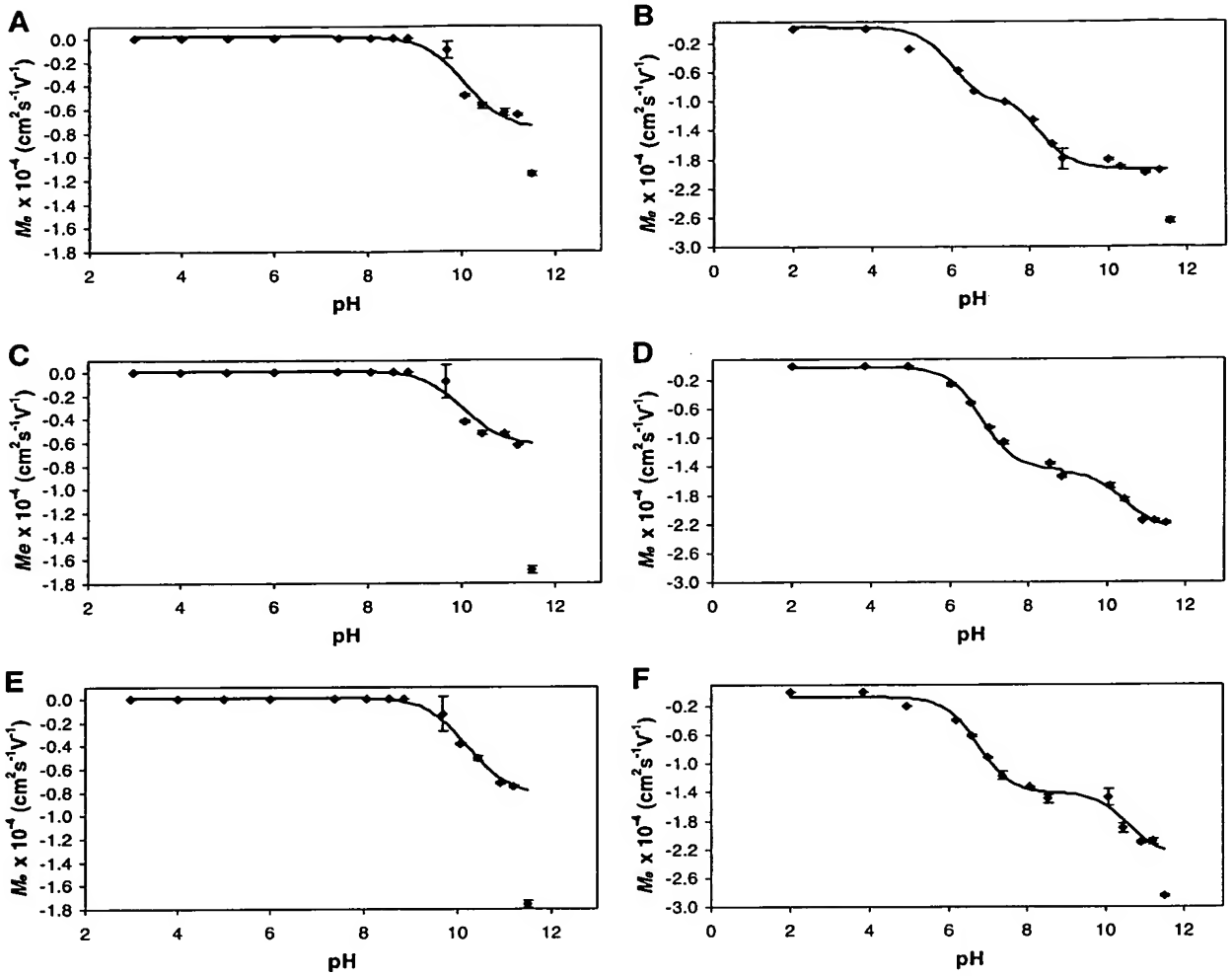

Figure 2. Ionisation curves ($n = 3$) obtained for the test compounds by capillary electrophoresis (A, diosmin; B, diosmetin; C, hesperidin; D, hesperetin; E, naringin; F, naringenin).

Table 1. Ionisation constants and absolute mobilities of mono (M_a^-) and double charged (M_a^{2-}) ions experimentally determined by capillary electrophoresis and estimated

	Diosmetin		Hesperetin		Naringenin	
	Experimental[a]	Estimated[b]	Experimental[a]	Estimated[b]	Experimental[a]	Estimated[b]
pK_{a1}	6.0 ± 0.1	7.29	6.8 ± 0.1	7.27	6.8 ± 0.1	7.27
M_a^-	−1.0E−4		−1.5E−4		−1.5E−4	
pK_{a2}	8.2 ± 0.2	9.54	10.4 ± 0.2	9.78	10.4 ± 0.2	9.47
M_a^{2-}	−1.9E−4		−2.2E−4		−2.3E−4	
pK_{a3}	>11.50	11.28	>11.50	11.31	>11.50	11.31
	Diosmin		Hesperidin		Naringin	
pK_{a1}	10.1 ± 0.2	9.39	10.0 ± 0.2	9.56	10.2 ± 0.1	9.34
pK_{a2}	10.1 ± 0.2	10.07	10.0 ± 0.2	10.17	10.2 ± 0.1	9.94
M_a^{2-}	−6.5E−5		−5.4E−5		−7.6E−5	
pK_{a3}–pK_{a8}	>11.50	⩾12.36	>11.50	⩾12.36	>11.50	⩾12.52

[a] Sigmoidal adjustment of CE mobilities against pH (GraphPad Prism 5).
[b] MarvinSketch (http://www.chemaxon.com/marvin).

For the flavonoids studied, considering the number of ionisable OH groups present in each molecule, three pK_a values for the aglycones were expected (corresponding to the three phenolic OHs) and eight for the glycosides (two phenolic and six alcoholic OHs), as estimated using MarvinSketch program (Table 1).

However, since dissociation of the alcoholic OHs occurs only in strongly basic conditions, which are outside of the experimental pH range of this methodology, it was not possible to determine them. The curves obtained from the experimental data (Fig. 2), for the glycosides, only show one inflexion point, (ca. pH 10.0) and a second one is suggested at higher pH values (>11.5). The first mobility differential probably results from a doubly charged ion since the experimental data does not fit perfectly to Eq. (2) in the case of hesperidin and diosmin. In fact, the experimental curves are steeper than the adjusted ones in which the slope is constricted to 1. This is indicative of a faster increase in the effective mobility against pH, that is not consistent with the formation of a single charged ion. These results were expected since the predicted difference between pK_{a1} and pK_{a2} is less than one unit. Naringin afforded a better curve fit but since the determined absolute mobility (M_a) is in the same order of magnitude of those obtained for hesperidin and diosmin it is likely that it corresponds to a double charged ion as well. The better fit for naringin curve was probably resulted from the difference between pK_{a1} and pK_{a2}. The second mobility increase at pH values >11.5, which cannot be adjusted due to insufficient experimental data at higher pH, must correspond to the ionisation of the OH groups of the carbohydrate.

For the aglycones diosmetin and naringenin, as expected, three pK_a values were detected but only two could be accurately determined since the third inflexion point occurred at high pH values (>11.5). For hesperitin only two mobility steps were detected in the experimental pH range but the M_a and slopes of the curves are consistent with a single deprotonation in each case.

In the aglycones, pK_{a1} should correspond to the ionization of the 7-OH group since the negative charge resulting from deprotonation of this group will be stabilized by conjugation.

The deprotonation of the hydroxyl groups in ring B (3′-OH or 4′-OH) should correspond to pK_{a2} (pK_{a1} in the case of the glycosilated flavonoids) since deprotonation of the 5-OH group must be the hardest to occur due to hydrogen bonding with the vicinal carbonyl group. Also, the introduction of a second negative charge in ring A, in the case of the aglycones, would make it more unstable. This is also the reason why deprotonation of 5-OH in the aglycones (which corresponds to pK_{a3}) occurs at higher pH than in the glycosilated flavonoids (pK_{a2}) where position 7 is not ionizable.

2.2. Transepithelial transport experiments

Based on Caco-2 cell viability the highest concentration showing reduced cytotoxic effect (under IC_{50}) for all compounds was chosen for the transport studies (15 μM).

The stabilities of the donor solutions in Hank's Balanced Salt Solution (HBSS) at this concentration were studied. Samples analysed by HPLC at different times after preparation revealed that, after two hours, over 80% of the original amount of each compound remained in solution, however, after 24 h, no diosmin and only about 50% diosmetin was still in solution (Table 2). The permeability assays of diosmin and diosmetin were therefore performed under super-saturation conditions, however since the permeability assays take only two hours, the amount of compound remaining in solution during this period permitted the evaluation of permeability.

No transport across the cell layer or metabolism was detected in either direction with the glycosilated flavonoids, neither in the presence nor in the absence of glucose. The purpose of the assay in a glucose-free medium was to evaluate the possibility, suggested by other authors,[44,45] that glucose transporters could be responsible for the transport of flavonoid glycosides but this was not confirmed for the compounds in this study.

The aglycones permeated across the membrane to the acceptor compartments in both the apical to basolateral (AP–BL) and the basolateral to apical (BL–AP) assays. Figure 3A and B present the time course accumulation at the acceptor compartments in each case.

Based on the comparison of the ionisation curves of the aglycones and of the glycosides, it would be expected that permeation of the aglycones would be lower at the pH of the transport medium (7.4), because they are more extensively ionised. However opposite results were obtained and this is probably due to the aglycones' smaller size and higher lipophilicity as demonstrated by the octanol/water partition coefficients (log P) estimated with the software KowWin (http://www.syrres.com/esc/est_kowdemo.htm) (Table 3).

P_{app} values (Table 3) were obtained for the bidirectional transport of the aglycones using Eq. (3). The apparent permeabilities of the aglycones in the serosal direction ($P_{app,ab}$) were above 3×10^{-5} cm/s. This value is in the same order of magnitude of the apparent permeability of caffeine[32] which demonstrates that the compounds are highly permeable.

The $P_{app,ab}$ obtained for naringin and naringenin are in agreement with the ones found in the literature.[46]

Apparent permeabilities in the mucosal direction ($P_{app,ba}$) were lower than $P_{app,ab}$ for diosmetin and hesperetin while for naringenin $P_{app,ba}$ was slightly larger.

Table 2. Compounds (%) remaining in HBSS solution (15 μM) at selected time points after sample preparation

Flavonoid	20 min	60 min	120 min	180 min	24 h
Diosmin	102.8	n.d.	88.6	78.4	0.0
Hesperidin	99.8	n.d.	87.9	86.3	84.9
Naringin	98.3	n.d.	92.2	90.4	88.5
Diosmetin	100.1	96.5	101.2	98.4	50.9
Hesperetin	94.5	99.9	91.1	90.8	80.0
Naringenin	102.0	99.4	100.4	91.2	79.9

n.d., not determined.

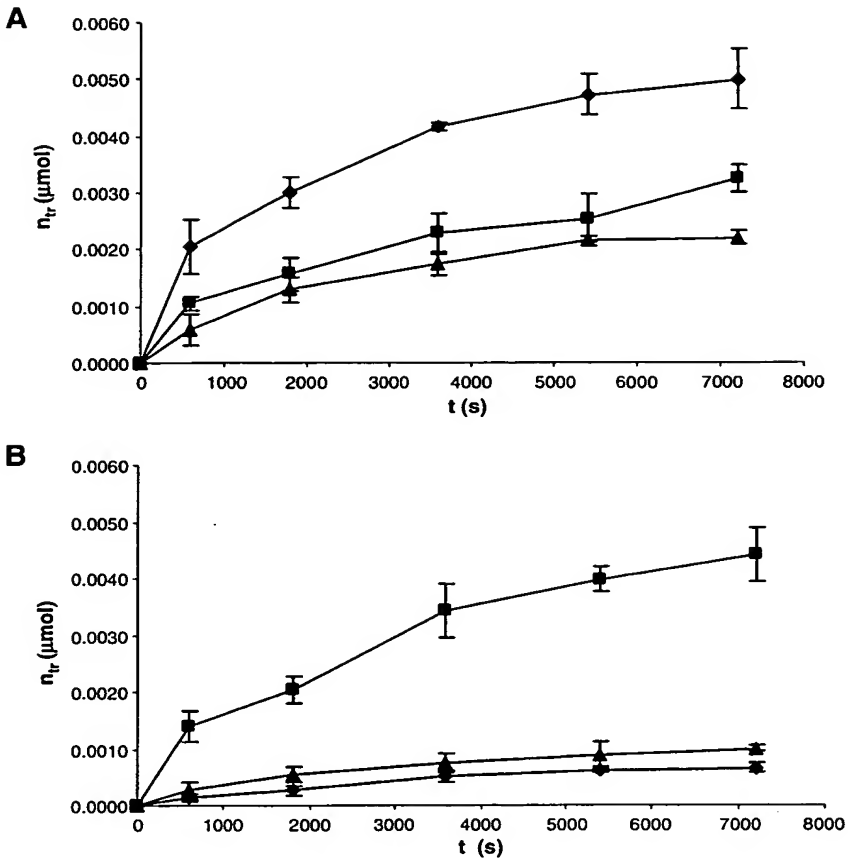

Figure 3. Cumulative amounts of the aglycones diosmetin (♦), hesperitin (▲) and naringenin (■) found in the receptor compartments in the apical-to-basolateral (A) and basolateral-to-apical (B) experiments. Error bars represent the confidence interval of three determinations.

Table 3. Apparent permeability values obtained for both AP–BL and BL–AP directions in the Caco-2 ($n = 3$) and in the PAMPA models ($n = 3$), molecular weights and estimated log P

Flavonoid	Caco-2 (AP–BL)		Caco-2 (BL–AP)		PAMPA		Log P[b]	MW
	P_{app} (10^{-6} cm/s)	Recovery (%)	P_{app} (10^{-6} cm/s)	Recovery (%)	P_{eff} (10^{-6} cm/s)	Recovery (%)		
Diosmin	[a]	61.9 ± 5.1	[a]	68.9 ± 3.1	[a]	n.d.	−0.72	608.6
Hesperidin	[a]	76.9 ± 7.4	[a]	80.2 ± 5.8	[a]	n.d.	−0.48	610.6
Naringin	[a]	85.7 ± 3.4	[a]	88.4 ± 7.2	[a]	n.d.	−0.52	580.5
Diosmetin	76.2 ± 5.6	59.1 ± 5.5	12.4 ± 2.3	66.8 ± 4.6	76.1 ± 3.5	82 ± 30	2.67	300.3
Hespereti n	47.1 ± 9.4	34.1 ± 1.3	27.0 ± 6.2	70.1 ± 6.1	35.7 ± 1.4	84 ± 12	2.44	302.3
Naringenin	37.8 ± 7.3	54.4 ± 2.1	51.4 ± 5.9	93.0 ± 7.6	25.7 ± 2.7	87 ± 14	2.61	272.3

n.d., not determined.
[a] Transport not detected.
[b] Predicted with KowWin software.

P_{ratio} values were calculated from Eq. (4) in order to investigate possible efflux or active transport. It is generally assumed that a P_{ratio} greater than 2 is predictive of relevant efflux, that is, the drug is actively pumped back into the intestinal lumen thus reducing its intestinal absorption.[47] The P_{ratio} values were 1.4, 0.6, and 0.2, respectively, for naringenin, hesperetin, and diosmetin. It is therefore unlikely that an efflux mechanism is involved in the permeation of these compounds and there is also no evidence of active transport in the case of naringenin and hesperitin. On the other hand, for diosmetin, the permeability rate was five times larger in the serosal direction and therefore the possibility of active transport should not be excluded.

The mass balances, expressed as recovery percentage (also presented in Table 3) and determined from Eq. (5), were higher in the BL–AP experiments. The incomplete recoveries may be justified by partial precipitation, in the case of diosmin and diosmetin, and by accumulation

of the compounds in the cells, as reported by other authors for similar compounds.[48] Lipophilic compounds, like the aglycones in this study, may be considerably retained by the cell monolayer.

2.3. Metabolism evaluation

Low recoveries can also be due to metabolism which was in fact observed for the aglycones, more extensively when loading at the apical side. These results, together with the fact that recoveries were higher in the case of the BL–AP assay, may indicate that metabolism occurs preferentially at the apical membrane.

Metabolites, D1, D2, and D3 for diosmetin, H1, H2, and H3 for hesperetin, and N1 for naringenin, were detected in both compartments but at higher concentration at the apical side in the AP–BL assays (Fig. 4). These metabolites are likely to be conjugated glucuronides and sulfates. Furthermore, the retention times of some of the metabolites produced by Caco-2 cells, matched the retention times of the glucuronides synthesised by enzymatic glucuronidation reaction using UGT supersomes.

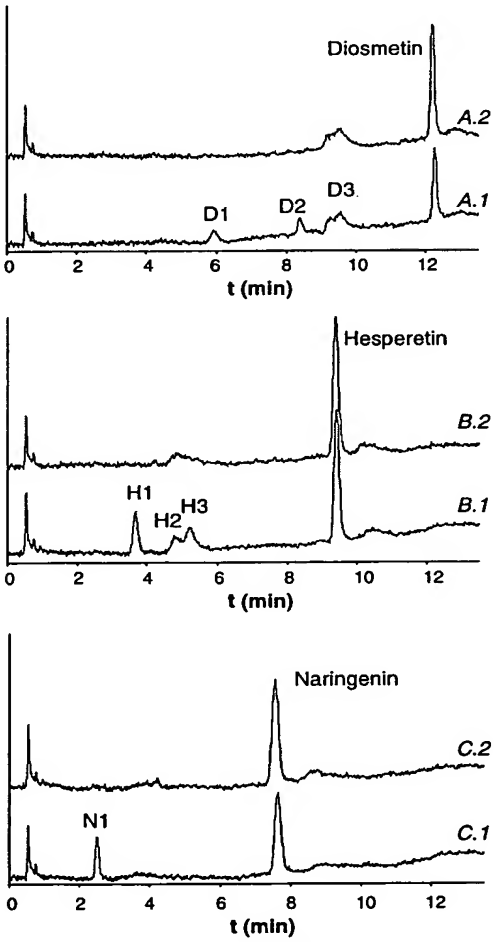

Figure 4. Metabolites of diosmetin, hesperetin and naringenin detected by HPLC in the apical solution after 120 min of apical loading (A.1, B.1, and C.1) and corresponding solutions after enzymatic hydrolysis (A.2, B.2, and C.2). Conditions as described in Experimental.

Confirmation was achieved by incubation of the samples obtained from the transport experiments with β-glucuronidase, which converted some of the metabolites back to the corresponding aglycones: D1, D2, H1, and N1 disappeared after incubation with β-glucuronidase from bovine liver while, at the same time, the peaks of the aglycones increased. H2 and D3 peaks were not affected.

2.4. Permeability studies through artificial membrane

In contrast with the aglycones, the glycosides were not transported across the soy lecithin lipidic membrane in the PAMPA model (Table 3). Soy lecithin was chosen as the phospholipidic component of the membrane because it has been referred to as affording the best correlations with human jejunal permeabilities.[49] In addition, the higher negative charge content of this type of membrane in comparison to other membranes such as egg lecithin can be an advantage for the transport of the compounds studied: at the pH used in these assays, the flavonoids are partially charged, and this may increase the repulsions between the sample and the negatively charged phospholipids, preventing H-bonding or other intermolecular forces, and consequently decreasing membrane retention.[49]

The effective permeabilities (P_{eff}) determined by this method predicted the highest rate of absorption for diosmetin, followed by hesperitin and then naringenin, which is in agreement with the data obtained using the Caco-2 model providing some evidence of passive transport. Membrane retention was less then 20% for all compounds.

Naringenin glucuronides obtained by enzymatic reaction were also tested in the PAMPA model but no transport was detected as expected due to their probable ionised state at the test pH.

3. Conclusions

To the best of our knowledge this is the first time that experimental ionization constants have been determined for diosmin, hesperidin and their aglycones. The pK_a obtained by CE in this study for naringin and naringenin are in good agreement with the ones found in the literature.[36,37]

Hesperidin, diosmin and naringin did not permeate, confirming other author's observations of low permeabilities for flavonoids glycosides and indicating that hydrolysis by intestinal microflora glycosidases may be necessary before absorption through the intestinal membrane.

The aglycones are more extensively ionized at physiological pH than the corresponding glycosides; however, the permeability results for the three aglycones studied,

that is, diosmetin, hesperetin and naringenin demonstrated that they are better absorbed through the intestinal epithelium. Absorption is most probably achieved passively for hesperitin and naringenin since bidirectional permeabilities were in the same order of magnitude, however in the case of diosmetin, the hypothesis of active transport should not be disregarded since P_{app} was significantly lower in the mucosal direction. On the other hand, the results obtained with the Caco-2 and the PAMPA models correlated well providing some evidence of passive transport. More experiments are needed in order to clarify this. The aglycones were moderately metabolized during the Caco-2 permeability experiments and therefore pre-systemic conjugation of the aglycones may be a relevant factor for limited bioavailability, unless metabolites are re-hydrolyzed.

4. Experimental

4.1. Test compounds

Diosmin (96.6%), hesperidin (94.5%), hesperetin (96.0%) were from Sigma–Aldrich (St. Louis, MO, USA). Diosmetin and naringenin (90%) were obtained from Extrasynthése (Genay, France). Naringin (95%) was from Fluka (Buchs, Switzerland).

Stock solutions of each flavonoid were prepared in DMSO (99.5%, Lab-Scan, Dublin, Ireland) at about 500 μM.

4.2. Determination of dissociation constants

Analyses were carried out in a Beckman P/ACE MDQ capillary electrophoresis system coupled with a diode array detector (DAD) (Palo Alto, CA, USA). A 75 μm, 50 cm (40 cm to detector) fused silica capillary was used and maintained at 25 °C.

The mobilities were determined using running buffers with an ionic strength of 0.05 and pH in the range of 2.0–11.5, prepared as described in Table 4. Sodium dihyrogenphosphate (99%, Merck, Darmstadt, Germany), sodium hydrogenphosphate (99%, Riedel-de Haën, Seelze, Germany), *ortho*-phosphoric acid (85%, Panreac, Castellar del Vallès, Spain), potassium hydrogenphosphate (99%, Sigma–Aldrich, Steinheim, Germany), potassium phosphate tribasic (≥98%, Sigma–Aldrich, Steinheim, Germany), sodium acetate (99%, Riedel-de Haën, Seelze, Germany), acetic acid (99.79%, Fisher Scientific Ltd., Loughborough, UK), ammonia (25%, Panreac, Castellar del Vallès, Spain), ammonium chloride (>99.5%, Merck, Darmstadt, Germany), Trizma base (Sigma–Aldrich, Steinheim, Germany) and Trizma HCl (99%, Sigma–Aldrich, Steinheim, Germany) were used to prepare the buffer solutions. Sample solutions were prepared by dilution from stock solutions in water. Dimethyl sulfoxide (DMSO) was used as neutral marker (1%).

Table 4. Composition of running buffers used for the determination of the pK_a values by CE (I = 0.05 M)

pH range	Constituent	Stock solutions
2.5–3.0	Phosphate	0.5 M H_3PO_4, 1.0 M NaH_2PO_4
4.0–5.0	Acetate	1.0 M CH_3COONa, 1.0 M CH_3COOH
6.0–7.5	Phosphate	1.0 M NaH_2PO_4, 0.5 M Na_2HPO_4
8.0–9.0	Tris	0.2 M Tris, 0.2 M Tris.HCl
9.2–10.5	Ammonium	0.1 M NH_3, 0.1 M NH_4Cl
11.0–11.5	Phosphate	0.1 M K_3PO_4, 0.5 M K_2HPO_4

Prior to the assay of each compound, the capillary was rinsed with 0.1 mM NaOH, followed by water and the more basic running buffer. Mobilities were then determined in this buffer. Before proceeding to the determination of mobility with the buffer immediately following in the pH scale, the capillary was rinsed with it for 5 min. Injection was made by pressure (100 mBar) for 6 s and a potential of 20 kV was applied throughout the run.

The determination of ionisation constants by CE is based on the relationship between the electrophoretic mobility and the degree of dissociation of a species over a range of electrolyte pHs. The effective mobility, M_e ($cm^2\ s^{-1}\ V^{-1}$), of an ionic species at a particular pH, defined as the difference between the apparent mobility (M_{app}) and the mobility due to the electroosmotic flow (M_{EOF}), can be calculated using the following equation:

$$M_e = M_{app} - M_{EOF} = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (1)$$

where L_c is the length of the capillary to the detector (cm), L_d is the total capillary length (cm), V is the applied voltage (V) and t_{app} and t_{EOF} are the migration times (s) of the analyte and a neutral marker compound. For dilute solutions, a plot of effective mobility of an ionic species against the pH of the running buffer affords a sigmoidal curve (2) whose inflection point corresponds to the analyte pK_a.[40]

$$M_e = \frac{M_a - M_o}{1 + 10^{(pH - pK_a)}} + M_o \quad (2)$$

M_a represents the absolute mobility and M_o represents the mobility of the unionized species which is zero.

Each sample was tested three consecutive times at each pH and the mobilities were calculated based on the migration time of the sample and of the t_{EOF}, using Eq. (1).

The average of the mobilities of each sample was fitted by non-linear regression to a sigmoidal curve (2) using GraphPad Prism software (San Diego, CA 92130, USA).

Since the test compounds are multiprotic and the micro ionisation constants of the different ionisable groups in each molecule are quite close, it was only possible to determine the apparent ionisation constants. Whenever more than one inflexion point was distinctively determinable, the non-linear model used was run independently on different sets of data containing each inflexion point of the curves.

The micro pK_as of the test compounds were also estimated by molecular simulation using the software MarvinSketch.

4.3. Transepithelial transport experiments

Caco-2 cells from the German Collection of Microorganisms and Cell Cultures (DSMZ) were grown in RPMI-1640 media supplemented with L-glutamine and 10% fetal bovine serum (FBS), all from Gibco Invitrogen (Gran Island, NY, USA), at 37 °C in culture flasks (Nunc, Roskilde, Denmark) in a humidified atmosphere with 5% CO_2.

Before conducting the transport studies, the cytotoxicity of each test compound was examined at different concentrations using the CellTiter 96® AQueous Assay from Promega (Madison, WI, USA), which is composed of solutions of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine methosulfate (PMS).

This method is based on the capacity of viable cells to convert a tetrazolium salt (MTS) into aqueous soluble formazan in the presence of phenazine methosulfonate (PMS) which acts as an electron coupling agent. The quantity of formazan product, proportional to the number of living cells in culture, is measured by absorbance at 490 nm.

Cells were seeded in a 96-well plate at a concentration of 1.0×10^4 cells/cm^2 and incubated until they reached confluence. Then they were incubated for 2 h with flavonoid solutions in concentrations ranging from 0.5 to 100 μM prepared in culture medium, diluting from the stock solutions. The combined solution of MTS/PMS was prepared as described in the technical bulletin provided with the assay solutions. Then 40 μL were added to each well of the assay plate containing 200 μL of culture medium. After 2 h of incubation the absorbance at 490 nm was measured using a microplate spectrophotometer (Molecular Devices, SpectraMax Plus^{384}).

For transport studies, cells were grown in Transwell inserts (polycarbonate membrane, 12 mm diameter, 0.4 μm pore size, Corning Costar, NY) at a density of 1.0×10^5 cells/cm^2. The inserts were housed in 12-well plates containing culture buffer. Cells were used at passage 23–28, 21–23 days after seeding.

The integrity of Caco-2 monolayers was monitored routinely over the growing period, by measuring the Transepithelial Electrical Resistance (TEER) with an EVOM Epithelial Tissue Voltammeter (World Precision Instruments, USA). Inserts were used for transport experiments only when the values of TEER exceeded 400 Ω cm^2. TEER was also monitored after the transport experiments to confirm membrane integrity and it was over 400 Ω cm^2 in all cases.

The transport studies were conducted at 37 °C, replacing the culture medium by the transport medium (HBSS) in the presence or absence of glucose, consisting of 1.3 mM $CaCl_2$ (95%, Absolve), 5.4 mM KCl (99.5%, Riedel-de Haën, Seelze, Germany), 0.44 mM KH_2PO_4 (≥95%, Riedel-de Haën, Seelze, Germany), 0.49 mM $MgCl_2$ (≥98% Merck, Darmstadt, Germany), 0.41 mM $MgSO_4$ (99.5%, May & Baker, Dagenhan, England), 137 mM NaCl (≥99.5%, Fluka, Buchs, Switzerland), 0.34 mM Na_2HPO_4 (≥99%, Riedel-de Haën, Seelze, Germany), 5.5 mM D-glucose (≥99%, Fluka, Buchs, Switzerland), and 4.2 mM $NaHCO_3$ (99.95 %, Fisher Scientific Ltd., Loughborough, UK) (pH 7.4).

Experiments were performed in triplicate in the AP–BL and BL–AP directions. The cell layers were washed twice for 30 min with warm HBSS before every experiment and then 15 μM solutions of each flavonoid, prepared in the transport media by dilution from the stock solutions, were added either to the apical or to the basolateral compartment. Samples were taken from the acceptor compartment, at selected times, ranging from 10 to 120 min, replacing always with an equal volume of HBSS. The solution at the donor compartment was also collected at the end of the experiment. Since it has been previously suggested[44,45] that glucose transporters could be responsible for the transport of flavonoid glycosides, an AP–BL assay in free-glucose medium was also conducted.

The P_{app} of each compound, expressed in cm/s, was determined according to the following equation:

$$P_{app} = J/A_S C_0 \quad (3)$$

where J is the rate of appearance of the compound on the acceptor compartment (μmole/s), C_0 is the initial concentration on the apical side (mM), and A_S is the surface area of the monolayer (cm^2).[50]

The permeability ratios (P_{ratio}) were calculated according to[47]:

$$P_{ratio} = P_{app,\,ba}/P_{app,\,ab} \quad (4)$$

The mass balance, expressed as the recovery percentage (R%), was determined according to the equation:

$$R\% = 100 \cdot (C_{a,\,120\ min} V_a + C_{d,\,120\ min} V_d)/(C_{d,\,0\ min} V_d) \quad (5)$$

where $C_{a,\,120\ min}$ and $C_{d,\,120\ min}$ are the concentrations measured at the end of the assay (120 min) in the acceptor and donor compartment, respectively; $C_{d,\,0\ min}$ is the concentration in the donor compartment at t_0; V_a and V_d are the volumes of the acceptor and donor compartments, respectively.[51]

4.4. Permeability studies through artificial membrane

The permeation studies through artificial membrane were done using the PAMPA model. This model consists of a 96-well microtitre plate (*p*ION, Woburn, MA, USA), which serves as the donor chamber, and a 96-well filter plate (MultiScreen-IP, Millipore), that serves as the acceptor compartment. The acceptor plate, placed directly on the donor plate, is bottomed by a hydrophobic microfilter disc (Immobilion-P membrane,

0.45 μm), impregnated with a phospholipid solution. A non-polar solvent such as *n*-dodecane is often used to dissolve phospholipids before applying on the filter.[52] In these tests, 5 μL of a 20% (w/v) solution of soy lecithin (Centrolex-R, Central Soya) in dodecane (98%, Fluka, Buchs, Switzerland) were used as phospholipidic membrane. Assays for each of the flavonoids were performed in triplicate by the double-sink method previously described.[49] Briefly, the method consists in two sets of experiments, one using a pH gradient between the acceptor and the donor reservoirs and the other using the same pH in both compartments. In the gradient experiment, a surfactant is added to the acceptor solution to create sink conditions while in the iso-pH experiment, a surfactant is added to the donor solution.

Acceptor and donor buffers were prepared with Na_2H-PO_4 and NaH_2PO_4, to obtain a final phosphate concentration of 10 mM and the ionic strength was set at 154 mM by adding NaCl. For the iso-pH assay, solutions were at pH 7.4 in both compartments. The donor solution also contained the test compounds and 35 mM sodium dodecyl sulfate (SDS). For the pH gradient assays the buffer at pH 7.4 containing 35 mM SDS was used as acceptor solution while the donor solutions consisted of the test compounds in pH 6.8 buffer. In both assays, the concentrations of the flavonoids were 15 μM, prepared from dilution of the stock solutions and all solutions contained 10% DMSO. The model was placed in a minishaker (IKA) with a microtitre plate support which was set to 400 rpm. After 24 h the two plates were separated, the acceptor and the donor solutions were analyzed by HPLC and effective permeability was calculated according to the iterative method described by Avdeef.[49]

Permeation of naringenin glucuronide was also tested.

4.5. Identification of metabolites

For enzymatic synthesis of aglyones' glucuronides, Human UGT1A3 BD Supersomes Enzyme supplied with the corresponding UGT reaction Mixtures from BD Biosciences (San Jose, CA USA) was used as described in the technical bulletin provided with the assay solutions. In brief, a 0.2 mL reaction mixture containing 1 mg/mL protein, 2 mM uridine diphospho-glucuronic acid (UDPGA), 10 mM magnesium chloride, 0.025 mg/mL alamethicin, 50 mM Tris–HCl (pH 7.4) and 0.3 mM of the substrate was incubated at 37 °C. For naringenin and hesperetin the incubation time was around 7 h and for diosmetin 27 h. After incubation, the reaction was stopped by the addition of 94% acetonitrile/6% glacial acetic acid and centrifuged (10,000*g*) for 3 min. The supernatant was analyzed by HPLC.

Samples collected from the apical side of Caco-2 inserts at the end of the transport experiments were used for the identification of metabolites. For testing the presence of glucuronide metabolites, the pH was adjusted to 4.5 with 100 μL of sodium acetate buffer 1 M. β-glucuronidase from bovine liver (Sigma–Idrich, Steinheim, Germany) was added (1500 U) and the samples were incubated at 37 °C.[28] Control samples were incubated in the absence of enzyme. After 24 h of incubation, the samples were analysed by HPLC.

4.6. Quantification by HPLC

Aliquots obtained from the different studies were analyzed by HPLC and concentrations determined by the external standard method. The HPLC system used was a Merck-Hitachi, consisting of a L-6200 intelligent pump, a L-4200 UV–vis detector, an AS-2000A autosampler and a L-5025 oven. The injection volumes were 30 μL and the detector wavelength was set at 275 nm. Separation was performed on a Chromolith RP-18e analytical column (4.6 mm × 100 mm) at 3.0 mL/min and 35 °C. Eluent A was composed of acetonitrile (HPLC grade, Lab-Scan, Dublin, Ireland)—acetic acid (99.7%, Panreac, Castelar del Vallès, Spain)—methanol (HPLC grade, Lab-Scan, Dublin, Ireland)—water (Milli-Q grade) (1:4:18:74, v/v/v/v) and eluent B was methanol. Gradient elution started at 100% A for four minutes and then graded to 25% B in seven minutes, staying at the final composition for one minute. Solutions for calibration curves were prepared by further dilution of the stock solutions with DMSO/MeOH (1:1) to reach concentrations in the range 0.5–20.0 μM.

Acknowledgment

The authors wish to thank *Fundação para a Ciência e Tecnologia* for a Grant (SFRH/BDE/15561/2005).

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Exhibit B

How should we assess the effects of exposure to dietary polyphenols in vitro?[1–3]

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ABSTRACT

Human intervention studies have provided clear evidence that dietary polyphenols (eg, flavonoids—eg, flavonols—and isoflavones) are at least partly absorbed and that they have the potential to exert biological effects. Biological activity of polyphenols is often assessed by using cultured cells as tissue models; in almost all such studies, cells are treated with aglycones or polyphenol-rich extracts (derived from plants and foods), and data are reported at concentrations that elicited a response. There are 2 inherent flaws in such an approach. First, plasma and tissues are not exposed in vivo to polyphenols in these forms. Several human studies have identified the nature of polyphenol conjugates in vivo and have shown that dietary polyphenols undergo extensive modification during first-pass metabolism so that the forms reaching the blood and tissues are, in general, neither aglycones (except for green tea catechins) nor the same as the dietary source. Polyphenols are present as conjugates of glucuronate or sulfate, with or without methylation of the catechol functional group. As a consequence, the polyphenol conjugates are likely to possess different biological properties and distribution patterns within tissues and cells than do polyphenol aglycones. Although deconjugation can potentially occur in vivo to produce aglycone, it occurs only at certain sites. Second, the polyphenol concentrations tested should be of the same order as the maximum plasma concentrations attained after a polyphenol-rich meal, which are in the range of 0.1–10 μmol/L. For correct interpretation of results, future efforts to define biological activities of polyphenols must make use of the available data concerning bioavailability and metabolism in humans. *Am J Clin Nutr* 2004;80:15–21.

KEY WORDS Polyphenols, flavonoids, isoflavones, phytochemicals, plant bioactives, antioxidants, human metabolism, first-pass metabolism, conjugation, quercetin

INTRODUCTION

Polyphenols have been shown, in both in vitro test systems and small animal models, to induce responses consistent with the protective effects of diets rich in fruit and vegetables against degenerative conditions such as cardiovascular disease (CVD) and cancer (1, 2). In fact many polyphenols, particularly flavonoids (eg, flavonols) and isoflavones, showed potent bioactivity when tested in vitro, which led to clinical trials assessing them with respect to a variety of effects (3, 4). However, because clinical studies are expensive and time-consuming, it is also necessary to optimize the use and interpretation of in vitro experiments.

Human tissues are exposed to polyphenols via the blood, which is the only route through which dietary polyphenols can reach tissues and their cells, except for the cells lining the intestinal tract. Understanding that polyphenols are substantially modified during absorption and identifying the physiologically relevant conjugates are essential to the planning of meaningful in vitro studies of polyphenol activity. Some controversy has attended hypotheses about the nature of circulating conjugates for particular polyphenols, but recent improvements in the analytic methods have resolved many of these questions. In the past few years, studies using physiologic concentrations of polyphenol conjugates helped clarify their specific mechanisms of action in vivo and advanced the field of understanding polyphenols in relation to health. In this article, we briefly discuss the arguments for using physiologic polyphenol conjugates to assess biological responses in vitro, and we define both what is known about polyphenol conjugates in vivo and where the gaps are in our knowledge of this subject.

HOW ARE POLYPHENOLS METABOLIZED?

The metabolism of several common polyphenols is now reasonably well understood. An important fact is that polyphenols are extensively altered during first-pass metabolism so that, typically, the molecular forms reaching the peripheral circulation and tissues are different from those present in foods (5–10). The term *metabolism* is used here to describe the typical modifications that occur during or after absorption. In general, the resulting metabolites are conjugates (eg, sulfates and glucuronates) of the parent aglycone or conjugates of methylated parent aglycones. Catabolism of polyphenols in humans usually occurs only as a result of microbial activity in the (large) intestine.

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Received October 24, 2003.

Accepted for publication January 16, 2004.

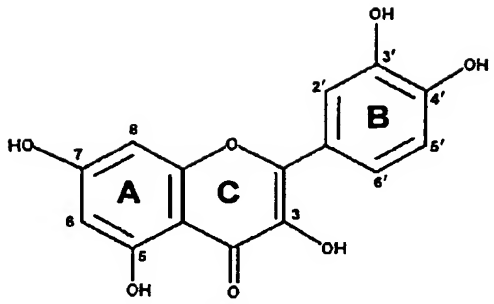

FIGURE 1. The structure of quercetin (3,3′,4,5,7-pentahydroxyflavone). Quercetin contains 5 hydroxyl functional groups that have the potential to be conjugated and that differ in their inherent chemical reactivities (3 > 7 > 3′ > 4′ ≫ 5). Quercetin in human plasma is found as sulfate and glucuronate conjugates, and conjugation occurs at positions 3, 3′, and 4′, but not at position 5 or 7. Methylation of the catechol function (3′/4′-dihydroxy in the B-ring) also occurs, which gives rise to methylated conjugates.

Most polyphenol glucosides are deglycosylated by β-glucosidases in the small intestine, namely, the broad-specificity cytosolic β-glucosidase and lactase phlorizin hydrolase; this step is requisite for the absorption of many of these polyphenols (*see*, for example, 11, 12). After absorption, flavonoids are metabolized by the phase II drug–metabolizing enzymes, the uridine-5′-diphosphate glucuronosyl-transferases, sulfotransferases, and catechol-*O*-methyltransferases. The resulting molecules are glucuronate and sulfate conjugates with or without methylation across the catechol functional group, and many are multiply conjugated (**Figure 1**). The small intestine appears to be the organ primarily responsible for glucuronidation, but it also has a role in methylation (13, 14). The major products of small-intestine metabolism in the hepatic portal vein are glucuronides and perhaps methylated glucuronides. The conjugates may then gain access to hepatocytes and may be further methylated, glucuronidated, or sulfated therein (13, 15).

It is instructive to compare the fates of dietary polyphenols and of (oral) pharmaceuticals. Most drugs are designed or selected to be relatively slowly metabolized, and they are generally delivered at a dose high enough that most of the dose escapes first-pass metabolism. This ensures that a sufficient amount of the active drug (in the unmetabolized, unconjugated form) is delivered to the appropriate tissues. In contrast, polyphenols delivered through human diets are at low doses, and, in most cases, they do not escape first-pass metabolism (16). The net result of the extensive first-pass metabolism of dietary polyphenols is that, with only a few exceptions, the predominant (and very often exclusive) forms in plasma are conjugates (glucuronates or sulfates, with or without methylation). These conjugates are chemically distinct from their parent compounds, differing in size, polarity, and ionic form. Consequently, their physiologic behavior is likely to be different from that of the native compounds. Glucuronates and sulfates are also negatively charged at physiologic pH. Therefore, to assess in vitro (eg, by using cultured cells) the possible contribution of polyphenols to the overall protection against degenerative diseases afforded by diets rich in fruit and vegetables and to define mechanisms, it is crucial that in vitro studies are designed to use relevant conjugates found in vivo.

Although the processes of glucuronidation, sulfation, and methylation are now well established and accepted, there are numerous sites of possible conjugation, and recent efforts have focused on identifying specific structures that exist in vivo.

IDENTIFYING STRUCTURES OF PLASMA POLYPHENOLS IS KEY TO DEFINING THEIR BIOLOGICAL ACTIVITIES IN HUMANS

Advances in the understanding of polyphenol metabolism have been made possible by improvements in the analytic methods used, particularly the use of mass spectrometry in combination with high-resolution chromatography systems (especially reversed-phase HPLC) and with detection systems such as mass spectrometry, coulometric electrochemical, and diode array. Whereas most studies up to the middle or late 1990s measured total aglycones in plasma and urine after chemical or enzymatic deconjugation, or both (eg, 17), several studies now report the polyphenol conjugate composition of human plasma or urine samples after the ingestion of polyphenols or polyphenol-rich foods.

The conjugates and approximate concentrations of common dietary polyphenols present in vivo after oral consumption of a physiologically relevant amount of a common dietary source are summarized in **Table 1**. Although it has been established that products of microbial transformation (eg, ring-fission products) form in humans and that they are excreted in urine, those products have not been included because they (eg, hydroxylated phenylacetic acids and hippuric acid) are common to many polyphenols, and there is little information regarding their biological activity.

The flavonoid quercetin (flavonol subclass) is one of the most extensively studied polyphenols. It serves as a good example here because its metabolism in humans is well understood, and many conjugates have been identified. Flavonols are found in foods mainly as glycoside conjugates. The flavonol conjugates that have been identified in plasma and urine from persons fed quercetin-containing foods are not those found in food. For example, plasma samples from volunteers receiving quercetin orally (as an onion meal, buckwheat tea, or pure quercetin, quercetin-4′-glucoside, quercetin-3-glucoside, or quercetin-rutinoside supplements) contained conjugated forms of quercetin but not quercetin glucosides, quercetin rutinoside, or quercetin aglycone (6, 7, 18, 52, 53). Day et al (6) showed that the plasma of volunteers fed fried onions (containing quercetin-4′-glucoside and quercetin-3,4′-di-glucoside) contained a mixture of glucuronidated and sulfated conjugates of quercetin and methylquercetin. A total of 12 discrete quercetin conjugates were detected, and several were identified by using a combination of retention time, ultraviolet spectra, shift reagents, mass spectrometry, and comparison with authentic standards. Three of the 4 major conjugates of quercetin in plasma were identified positively as quercetin-3-glucuronide, 3′-methylquercetin-3-glucuronide, and quercetin-3′-sulfate, respectively. The fourth major conjugate was identified as one of several isomeric quercetin diglucuronides. Evidence was also obtained for the presence in human plasma of lower concentrations of quercetin-3′-glucuronide and 3′-methylquercetin-4′-glucuronide.

The metabolisms of most other dietary polyphenols in humans are comparable in several ways: (*1*) glycosides are generally not found in plasma or urine in the form ingested, (*2*) the major forms in plasma and urine are sulfate and glucuronate conjugates of the parent aglycones, (*3*) methylation may occur on polyphenols that contain orthohydroxy functional groups, and (*4*) aglycones are

TABLE 1
Summary of evidence for polyphenol structures in human plasma and urine

Polyphenol	Exact plasma or urine structures[1]	Evidence of conjugation[2]	Evidence of absence[3]
Flavonols			
Quercetin	Quercetin-3-glucuronide, 3′-methylquercetin-3-glucuronide, and quercetin-3′-sulfate in plasma at 0.1–1 μmol/L (5)	Mixture of glucuronides and sulfates of quercetin and methylquercetin in plasma (5, 7, 18, 19)	Quercetin, quercetin-3-glucoside, quercetin-4′-glucoside, and rutin (5, 7, 20)
Kaempferol	Kaempferol-3-glucuronide and free kaempferol present in plasma and urine; kaempferol-3-glucuronide in urine (21)		Phase I-mediated B-ring hydroxylation products such as quercetin; kaempferol-3-glucoside (21)
Flavones			
Luteolin	Not known	A luteolin glucuronide (22, 23)	
Chrysin	Chrysin-7-sulfate (major) and chrysin-7-glucuronide (minor) (24)		
Isoflavones			
Daidzein and genistein	Daidzein-7-glucuronide (54%), daidzein-4′-glucuronide (25%), daidzein 7- and 4′-sulfates (13%), daidzein-4′,7-diglucuronide (0.4%), daidzein sulfoglucuronides (0.9%), and unconjugated daidzein (7%) in urine, with similar profiles obtained for genistein (25)	Predominant forms in plasma and urine are sulfate, glucuronate, sulfoglucuronate, disulfate, and diglucuronate conjugates of daidzein, genistein, and microbial conjugates such as equol and *O*-desmethyl-angolensin (25–27)	Daidzein-7-glucoside (daidzein) and genistein-7-glucoside (genistin) (26)
Flavanones			
Hesperetin	Not known	Mixture of hesperetin glucuronides (87%) and sulfoglucuronides (13%) (28)	Hesperetin, hesperetin-7-rutinoside (hesperidin), and hesperetin sulfates absent from plasma (28)
Naringenin	Not known	Mixture of naringenin glucuronides and sulfates in plasma (29) and urine (30)	Naringenin absent from plasma (29) and from urine (31)
Flavanols[4]			
Catechin	Not known	Mixture of (+)-catechin sulfates, (+)-catechin sulfoglucuronide, and catechin-3′-glucuronide, with traces of 4′-methylated conjugates, in plasma and urine (31–33)	Catechin aglycone in plasma at <2 nmol/L after red wine consumption (34)
Epicatechin	Nonconjugated epicatechin (peak 0.15–0.22 μmol/L) in plasma. (−)-Epicatechin-3′-glucuronide, 4′-methyl-(−)-epicatechin-3′-glucuronide and 4′-methyl-(−)-epicatechin-5 or 7-glucuronide in urine (8)		Epicatechin aglycone not detected after green tea consumption (35)
Epicatechin gallate	Not known	Detected in plasma only after black tea consumption. Present exclusively as conjugates, but exact forms unknown (36)	Neither epicatechin gallate nor its conjugates detected in plasma or urine after green tea consumption (35,37)
Epigallocatechin	Nonconjugated (−)-epigallocatechin present in plasma at 0.08 μmol/L (35)		
Epigallocatechin gallate	Nonconjugated epigallocatechin gallate in plasma at 0.34 (38) or 0.14 (39) μmol/L		
Anthocyanins			
Delphinidin	Unchanged delphinidin glycosides at pmol/L–nmol/L concentrations (40–43)		
Cyanidin	Unchanged cyanidin glycosides at pmol/L–nmol/L concentrations (40–45)	Methylated and glucuronidated cyanidin glycosides (46)	
Malvidin	Unchanged malvidin glycosides at pmol/L–nmol/L concentrations (42)		
Petunidin	Unchanged petunidin glucoside at pmol/L–nmol/L concentrations (42)		
Peonidin	Unchanged peonidin arabinoside at pmol/L–nmol/L concentrations (42)	Glucuronidation of peonidin (48); presence of pelargonidin glucuronides and sulfates and mixed sulfoglucuronides (47)	

(*Continued*)

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TABLE 1 (*Continued*)

Polyphenol	Exact plasma or urine structures[1]	Evidence of conjugation[2]	Evidence of absence[3]
Pelargonidin	Unchanged pelargonidin-3-glucoside (47); conjugation position of glucuronides and sulfates not known		
Procyanidins			
B1, B2, B3, etc	Not known	Detection of procyanidin B1 and B2 in plasma after hydrolysis with glucuronidase and sulfatase (48, 49)	
Stilbenes			
Resveratrol	Very low concentrations (≈35 nmol/L) of unconjugated resveratrol with large oral dose (50)	Glucuronate and sulfate conjugates of resveratrol predominant in plasma after large oral dose (>98% of total, ≈2 μmol/L) (50)	

[1] Actual structures of the polyphenols in vivo (ie, in samples of plasma or urine or both). These data were derived from nonhydrolyzed samples, and the structural details were derived by using a combination of appropriate selective techniques such as HPLC with mass spectrometry, nuclear magnetic resolution spectrometry, sensitivity to hydrolysis by specific enzymes (eg, glucuronidase and sulfatase), and spectral shifts, usually in combination with authentic standards.

[2] Evidence of conjugation is usually obtained by comparing chromatograms before and after hydrolysis with β-glucuronidase or sulfatase enzymes, or both.

[3] These data indicate particular structures that have been investigated with the use of the approaches described above ([1]), but have been shown to be absent.

[4] The green tea sample and the urine samples were subjected to chiral analysis. The ratio of (+)-epigallocatechin to (−)-epigallocatechin in the green tea was 7:93. An identical result was obtained for the ratio of (+)-catechin:(−)-catechin in green tea. However, in urine after full hydrolysis, only the minus enantiomers [(−)-catechin and (−)-epigallocatechin] were detected. It is interesting that these are both 2,3-*cis* forms and are generally considered to be less stable thermodynamically than are the 2,3-*trans* forms (51).

absent or constitute only a very small proportion of the total amount of polyphenols present, except in green tea catechins, of which aglycones can constitute a significant proportion of the total amount in plasma (54). Furthermore, although isoflavones are usually glycosylated in foods (the exceptions are fermented soy products such as tempeh), a small but significant proportion (≈7%) exists in the plasma as aglycones (25), and the remainder is present as sulfate and glucuronate conjugates (10, 25, 26). Details are presented in Table 1. Originally, anthocyanins were thought to be an exception to item 2 above, because anthocyanin glucosides have been identified in human plasma and urine (*see* Table 1; 40–47), albeit at low concentrations (pmol/L–nmol/L range). In all but 2 of these reports (46, 47), the anthocyanin glycosides were the only form present in plasma or urine or both, and the urinary yield was extremely low (<0.05%). However, a recent report using improved methods and describing the conjugate profile of human urine after the ingestion of strawberries that contained pelargonidin glycosides showed that glucuronate and sulfate conjugates were the predominant structures (98% of total) and indicated a urinary yield of ≈2% (46). It is clear that the results from the earlier studies must be viewed with some caution because it is unlikely that pelargonidin would differ so dramatically from the closely related anthocyanins in the extent of its absorption and susceptibility to phase II metabolism.

EFFECT OF POLYPHENOL METABOLISM ON BIOLOGICAL ACTIVITY

What, then, are the effects of metabolism on the biological activities of quercetin? It has been shown that some conjugates of quercetin retain antioxidant properties and the ability to inhibit lipoxygenase and xanthine oxidase in vitro (55). Furthermore, quercetin glucuronides were shown to inhibit the *N*-acetylation of 2-aminofluroene (an arylamine carcinogen) by human acute myeloid leukemia HL-60 cells (56). Further studies show that quercetin-3-glucuronide (a major human conjugate of dietary quercetin) is able to prevent angiotensin-II–induced vascular smooth muscle cell hypertrophy in cultured rat aortic smooth muscle cells through its inhibitory effects on the JNK and AP-1 signaling pathways (57), possesses a substantial antioxidant effect on copper ion–induced oxidation of human plasma LDL as well as on 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity (58), and suppresses the peroxynitrite-induced consumption of lipophilic antioxidants in human plasma LDL (59). In general, the responses to quercetin conjugates were weaker than those to the aglycone. For example, the antioxidant activity of quercetin conjugates is, on average, about half that of aglycone, but there is significant variation according to the position of conjugation (55). Quercetin-3-glucuronide, one of the 3 major plasma quercetin conjugates, significantly delayed the Cu(II)-induced oxidation of human LDL ex vivo, but 2 other major conjugates (quercetin-3′-sulfate and 3′-methylquercetin-3-glucuronide) were largely ineffective (60). In contrast, the inhibition of JNK and AP-1 signaling pathways in rat aortic smooth muscle cells by quercetin-3-glucuronide occurred at concentrations similar to those of the aglycone (57).

In the aglycone form, quercetin is a powerful antioxidant in vitro (61). Indeed, in vitro studies using quercetin aglycone have shown its potential as an agent for the prevention or treatment (or both) of various cancers, CVDs, inflammation, dementia, and cataract. However, quercetin is highly unstable and is reactive at physiologic pH values (62, 63). Damaging effects, especially on kidney, were observed after very high doses of quercetin were given to volunteers intravenously (3), thus bypassing the protection afforded by the gastrointestinal epithelium and phase II conjugation. Such phase II conjugations disrupt the electron delocalization of the quercetin ring structure so that quercetin conjugates have a reduced tendency to undergo redox cycling, although their ability to function as antioxidants is not complete abolished. Fortunately, quercetin is present in foods almost exclusively as glycosides; onions, tea, and apples are the most important dietary sources (17, 54, 64). Very few foods contain

significant amounts of quercetin as aglycone; some red wines are a notable exception (65). Hence, dietary quercetin is unlikely to have a damaging effect on the body.

Although we have focused on quercetin as an example, it is worth mentioning the findings from some similar studies with other dietary polyphenols. Daidzein and genistein monoglucuronides are the major isoflavone conjugates in human plasma after ingestion of soy (66), and they possess some estrogenic properties but are weaker than those of their corresponding aglycones. With the exception of the gallate esters in green tea, orally delivered catechins appear in plasma predominantly (>98%) as conjugated forms (sulfated or glucuronidated conjugates, or both, with or without methylation; 31, 33–36, 67–69), and the major conjugates of (–)-epicatechin have been identified (*see* Table 1). It is noteworthy that the plasma conjugates (predominantly glucuronides and sulfates of (+)-catechin and methylated (+)-catechin) obtained after oral administration of pure (+)-catechin to rats effectively inhibited both the generation of reactive oxygen species and the binding of U937 monocyte cells to interleukin 1β–stimulated human aortic endothelial cells, whereas (+)-catechin did not do so (70).

IN VIVO POLYPHENOL CONJUGATES: FACT AND FICTION

The past few years have seen very significant advances in our understanding of polyphenol metabolism. However, controversy remains concerning the nature and properties of flavonoid conjugates in vivo, and that uncertainty hampers progress toward understanding the real contribution of flavonoids as dietary protective agents against cancer, CVD, and other diseases. The combination of very complex conjugate profiles, the difficulty in obtaining in vivo conjugates, and the seemingly endless variety of possible endpoints for the demonstration of biological effects provides significant challenges to those working in this scientific field. High-quality scientific data in this area are therefore extremely valuable. Conversely, conclusions drawn from poorly designed studies have the potential to be misleading.

Many reports describe in vitro bioactivity studies that used polyphenol aglycones, food, or herbal extracts. Some of these reports produced confusing or difficult-to-interpret results, especially if the reports claim to have identified a polyphenol as the much-searched-for chemopreventive agent for cancer, CVD, and other such illnesses or to have identified a health risk associated with polyphenols. The confusion and difficulty in interpreting results are more widespread when the reports are published in high-profile journals.

Also of concern are the growing numbers of reports in which the authors' claims of using physiologic conjugates are not supported by their own or any literature evidence. For example, Spencer et al (71) stated that their recent study used the major in vivo human conjugates of quercetin in cell culture to investigate the potential uptake of quercetin and assess cytotoxicity and cytoprotection in dermal fibroblasts. The authors stated, without evidence, that the major reported in vivo human conjugates of quercetin are quercetin-7-glucuronide and the aglycones 3′-methylquercetin (isorhamnetin) and 4′-methylquercetin (tamarixetin). We are not aware of any reports in the scientific literature that support this statement. Whereas the use of these conjugates for in vitro studies may be a step in the right direction, the biological activity of conjugates also may differ significantly among the positional isomers (55, 58, 72). It is critical that all future studies attempting to use in vitro models to assess the effects of polyphenols in humans use physiologic conjugates at appropriate concentrations.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Identification and measurement of the physiologic polyphenol conjugates are key prerequisites to an understanding of the role of dietary polyphenols in human health. Acquiring such data will permit more reliable investigation of many phenomena by using cost-effective in vitro models. In the long term, the application of advanced metabolomic approaches and nanotechnologies has the potential to significantly advance our understanding in this area.

We strongly recommend that all experiments using in vitro models to study biological responses to dietary polyphenols use only physiologically relevant flavonoids and their conjugates at appropriate concentrations, provide evidence to support their use, and justify any conclusions generated. When authors fail to do this, referees and editors must act to ensure that data obtained in vitro are relevant to what might occur in vivo.

All authors contributed to the writing of the manuscript. All contributors read and commented on the manuscript.

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The American Journal of Clinical Nutrition

Exhibit C

Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies[1–3]

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ABSTRACT

Polyphenols are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases is emerging. Bioavailability differs greatly from one polyphenol to another, so that the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues. Mean values for the maximal plasma concentration, the time to reach the maximal plasma concentration, the area under the plasma concentration-time curve, the elimination half-life, and the relative urinary excretion were calculated for 18 major polyphenols. We used data from 97 studies that investigated the kinetics and extent of polyphenol absorption among adults, after ingestion of a single dose of polyphenol provided as pure compound, plant extract, or whole food/beverage. The metabolites present in blood, resulting from digestive and hepatic activity, usually differ from the native compounds. The nature of the known metabolites is described when data are available. The plasma concentrations of total metabolites ranged from 0 to 4 μmol/L with an intake of 50 mg aglycone equivalents, and the relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the polyphenol. Gallic acid and isoflavones are the most well-absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins. Data are still too limited for assessment of hydroxycinnamic acids and other polyphenols. These data may be useful for the design and interpretation of intervention studies investigating the health effects of polyphenols. *Am J Clin Nutr* 2005;81(suppl):230S–42S.

KEY WORDS Polyphenols, flavonoids, isoflavones, flavonols, flavanones, hydroxycinnamic acids, hydroxybenzoic acids, anthocyanins, proanthocyanidins, catechins, bioavailability, metabolism, pharmacokinetics, elimination half-life, humans

INTRODUCTION

Epidemiologic studies have clearly shown that diets rich in plant foods protect humans against degenerative diseases such as cancer and cardiovascular diseases. Plant foods contain fiber, vitamins, phytosterols, sulfur compounds, carotenoids, and organic acids, which contribute to the health effects, but they also contain a variety of polyphenols, which are increasingly regarded as effective protective agents.

Polyphenols represent a wide variety of compounds, which are divided into several classes, ie, hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans. The chemical structures and the food contents of the various polyphenols have been reviewed elsewhere (1). One of the main objectives of bioavailability studies is to determine, among the hundreds of dietary polyphenols, which are better absorbed and which lead to the formation of active metabolites.

Many researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage. We have reviewed 97 studies of various classes of polyphenols, namely, anthocyanins, flavonols, flavanones, flavanol monomers, proanthocyanidins, isoflavones, hydroxycinnamic acids, and hydroxybenzoic acids. We have compiled the data from the most relevant studies, ie, those using well-described polyphenol sources and accurate methods of analysis, to calculate mean values for several bioavailability measures, including the maximal plasma concentration (C_{max}), time to reach C_{max}, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion. The results clearly show wide variability in the bioavailability of the different polyphenols.

ANTHOCYANINS

Anthocyanins are present in very large amounts in some diets. Servings of 200 g of aubergine or black grapes can provide up to 1500 mg anthocyanins and servings of 100 g of berries up to 500 mg. Therefore, an intake of several hundred milligrams would not be considered exceptional. The mean dietary intake in Finland has been estimated to be 82 mg/d, with the main sources being berries, red wine, juices, and the coloring agent E163 (M Heinonen, personal communication, 2001).

The results of a literature survey on the bioavailability of anthocyanins among humans are presented in **Table 1**. Single doses of 150 mg to 2 g total anthocyanins were given to the volunteers, generally in the form of berries, berry extracts, or concentrates. After such intakes, concentrations of anthocyanins

[1] From the Unité des Maladies Métaboliques et Micronutriments, INRA, Saint-Genès Champanelle, France (CMa, CMo, AS, CR), and the Nutrient Bioavailability Group, Nestlé Research Center, Lausanne, Switzerland (GW).

[2] Presented at the 1st International Conference on Polyphenols and Health, held in Vichy, France, November 18–21, 2004.

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Am J Clin Nutr 2005;81(suppl):230S–42S. Printed in USA.

TABLE 1
Bioavailability studies of anthocyanins or anthocyanin-containing foods[1]

Source	No. of subjects	Dose	T_{max} plasma	Plasma concentration	T_{max} urine (h)	Urinary excretion	Ref
			h	*nmol/L*	*h*	*% of intake*	
Black currant juice	17	20 or 12 mg total anth./kg bw	0.75	32–107[2]		0.045–0.072	2
Black currant juice (330 mL)	10	1 g total anth.	1	3.5–51[3]	1–1.5	0.032–0.046[3]	3
Black currant juice (200 mL)	4	153 mg total anth.			2	0.02–0.05/5 h[3]	4
Black currant concentrate	8	3.58 mg total anth./kg bw	1.25–1.75[3]	115 (4–60[3])	<4	0.06–0.11/8 h[3]	5
Elderberry extract (12 g)	4	720 mg total anth.				0.077/4 h	6
Elderberry extract (12 g)	4	720 mg total anth.	1.1–1.2[3]	97			7
Elderberry concentrate	16	1.9 g total anth.			1–2[3]	0.035/6 h	8
Spray-dried elderberry juice	7	500 mg total anth.			3–4[3]	0.01–0.04[3]	9
Freeze-dried blueberries	5	1.2 g total anth.	41	11–36[2]			10
Lowbush blueberries (190 g)	6	690 mg total anth.				0.004/6 h	6
Red wine (300 mL)	6	218 mg total anth.			6	1.5–5.1/12 h	11
Red wine (500 mL)	6	68 mg malvidin 3-glc	0.83	1.4	<3	0.016/6 h	12
Red grape juice (500 mL)	6	117 mg malvidin 3-glc	2	2.8		0.019/6 h	12
Red fruit extract (1.6 g)	12	2.7 mg cyan 3-glc/kg bw	1	29			13
Strawberries	6	77.3 mg pelargonidin 3-glc			2–4	1.8/24 h	14

[1] T_{max}, time to C_{max}; anth., anthocyanin; bw, body weight; glc, glucoside.
[2] Assuming average molecular weight of 465 g/mol for unit conversion.
[3] Depending on the anthocyanin considered in the mixture.

measured in plasma were very low, on the order of 10–50 nmol/L.The mean time to reach C_{max} was 1.5 h (range: 0.75–4 h) for plasma and 2.5 h for urine. Most studies reported low relative urinary excretions, ranging from 0.004% to 0.1% of the intake, although Lapidot et al (11) and Felgines et al (14) measured higher levels of anthocyanin excretion (up to 5%) after red wine or strawberry consumption. The time course of absorption was consistent with absorption in the stomach, as described for animals (15, 16). The most striking features of the survey were thus that anthocyanins are very rapidly absorbed and eliminated and that they are absorbed with poor efficiency.

Although anthocyanin bioavailability appears low, it could have been underestimated, for 2 main reasons, ie, some important metabolites might have been ignored or the methods used might need to be optimized for the analysis of anthocyanin metabolites. It is well known that different chemical forms of anthocyanins are present in equilibrium, depending on the pH. In most studies, analyses were performed with ultraviolet-visible light detection, on the basis of complete conversion of all of the chemical forms of anthocyanins into a colored flavylium cation with acidification. However, it is possible that some forms existing at neutral pH would not be converted into the flavylium form, because of putative binding to or chemical reactions with other components of the plasma or urine, for example. It would be very useful to have labeled anthocyanins for identification of all of the metabolites generated from these polyphenols.

With our current knowledge, there seem to be important differences in the metabolism of anthocyanins, compared with other polyphenols. Whereas flavonoids are generally recovered in plasma and urine as glucuronidated and/or sulfated derivatives, with no or only trace amounts of native forms, unchanged glycosides were the only metabolites identified for anthocyanins in most studies. However, glucuronides and sulfates of anthocyanins were recently identified in human urine with HPLC-mass spectrometry/mass spectrometry analyses (6, 14). In the study conducted by Felgines et al (14), monoglucuronides accounted for >80% of the total metabolites when analyses were performed immediately after urine collection. The authors also showed that all of the metabolites of the strawberry anthocyanins, except for the native glucoside, were very unstable and were extensively degraded when acidified urine samples were frozen for storage. This probably explains why such metabolites were not observed in previous studies. Therefore, it seems crucial to reconsider anthocyanin bioavailability, with methods that allow preservation of all of the metabolites in frozen samples.

Other metabolites that have not yet been considered but could contribute to the biological effects of anthocyanins are the metabolites produced by the intestinal microflora. However, studies performed in the 1970s showed that degradation of anthocyanins by the microflora occurs to a much more limited extent than with other flavonoids (17). Protocatechuic acid was identified as an abundant metabolite of cyanidin-3-*O*-glucoside in rats, but it was also formed in vitro with simple incubation of cyanidin with rat plasma in the absence of colonic bacteria (18). Identification of all of the microbial metabolites in humans should be reinvestigated with pure anthocyanins and not only berry extracts, which contain other polyphenols as well as anthocyanins.

FLAVONOLS

Flavonols, especially quercetin, have been extensively studied, mainly because they are widely distributed in dietary plants. However, their content in the diet is generally quite low. The daily intake of flavonols has been estimated as only 20–35 mg/d (19–22).

Twenty years after Gugler et al (23, 24) failed to find quercetin in plasma or urine from volunteers challenged with 4 g pure aglycone, the team of Hollman et al (23, 24) showed that quercetin was indeed absorbed in humans. They demonstrated that glucosides of quercetin were more efficiently absorbed than quercetin itself, whereas the rhamnoglucoside (rutin) was less efficiently and less rapidly absorbed (**Table 2**). This difference in absorption rates was confirmed by others (33, 34). When pure compounds were given, the bioavailability of rutin was ~20%

TABLE 2
Bioavailability studies of flavonols or flavanol-containing foods[1]

Source	No. of subjects	Dose	T_{max} plasma	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
			h	*μmol/L*	*μmol · h/L*	*% of intake*	*h*	
Pure quercetin	6	4 g		<0.33		<1		23
Onions	9 ileostomized	89 mg quercetin eq				0.31/13h		24
Pure rutin	9 ileostomized	100 mg quercetin eq				0.07/13h		24
Pure quercetin	9 ileostomized	100 mg quercetin eq				0.12/13h		24
Fried onions	2	64 mg quercetin eq	2.9	0.65			16.8	25
Onions	9	68 mg quercetin eq	0.7	0.74	7.7		28.0	26
Apples	9	107 mg quercetin eq	2.5	0.3	3.5		23.0	26
Pure rutin	9	100 mg quercetin eq	9.3	0.3	3.3			26
Complete meal	10	87 mg quercetin eq		0.37 at 3 h				27
Onions	5	186 mg quercetin eq	1.3–1.9	2.18		1.11		28
Onions	5	50 mg quercetin eq	2	0.83				29
Quercetin 4'-glucoside	9	150 mg	<0.5	3.5	18.8		21.6	30
Pure rutin	9	190 mg	6	0.18	3.7		28.1	30
Quercetin 3-glucoside	9	156 mg	0.6	5	19.1	3.6	18.5	31
Quercetin 4'-glucoside	9	160 mg	0.45	4.5	17.5	3.1	17.7	31
Pure rutin	3	500 mg	4–7	0.13–0.73				32
Pure quercetin	16	8, 20, 50 mg	2, 2.7, 4.9	0.14, 0.22, 0.29	1.74, 2.92, 3.77		17, 17.7, 15	33
Pure rutin	16	8, 20, 50 mg quercetin eq	6.5, 7.4, 7.5	0.08, 0.16, 0.30	1.26, 2.10, 3.36			33
Onions	12	100 mg quercetin eq	0.68	7.6	32.1	6.4	10.9	34
Pure quercetin 4'-glucoside	12	100 mg quercetin eq	0.7	7.0	27.8	4.5	11.9	34
Buckwheat tea	12	200 mg quercetin eq	4.3	2.1	12.6	1.0	10.3	34
Pure rutin	12	200 mg quercetin eq	7	1.1	8.3	0.9	11.8	34
Apple cider (1.1 L)	6	1.6 mg quercetin eq	0.66–1	0.14				35
Pure quercetin	12	0.14 mg/kg bw	0.5	0.15–0.42		2.9–7		36

[1] T_{max}, time to C_{max}; AUC, area under the curve; eq, equivalents; bw, body weight.

that of quercetin glucosides, on the basis of area under the plasma concentration-time curve values and relative urinary excretions (30, 34). The biochemical explanation for the better absorption of quercetin glucosides has been discussed elsewhere (1). It is clear that, for quercetin, bioavailability differs among food sources, depending on the type of glycosides they contain. For example, onions, which contain glucosides, are better sources of bioavailable quercetin than are apples and tea, which contain rutin and other glycosides.

The presence of intact glycosides of quercetin in plasma was debated a few years ago, but it is now accepted that such compounds are absent from plasma after nutritional doses (34, 37–40). Quercetin is not present as an aglycone and occurs only in conjugated forms. Generally, ~20–40% of quercetin is methylated in the 3'-position, yielding isorhamnetin (31, 34, 38). The exact nature of the metabolites present in plasma after the ingestion of onions was determined by Day et al (38). They identified quercetin-3-*O*-glucuronide, 3'-*O*-methylquercetin-3-*O*-glucuronide, and quercetin-3'-*O*-sulfate as the major conjugates.

Some phenolic and aromatic acids can also be produced from flavonols by the microflora. Quercetin degradation produces mainly 3,4-dihydroxyphenylacetic, 3-methoxy-4-hydroxyphenylacetic (homovanillic acid), and 3-hydroxyphenylacetic acid (17, 41–43). The total urinary excretion of microbial metabolites accounted for as much as 50% of the ingested dose among volunteers challenged with 75 mg rutin (44).

One characteristic feature of quercetin bioavailability is that the elimination of quercetin metabolites is quite slow, with reported half-lives ranging from 11 to 28 h. This could favor accumulation in plasma with repeated intakes. A few authors investigated the bioavailability of quercetin after several days or weeks of supplementation. Baseline quercetin concentrations, measured after overnight fasting, were generally ~50–80 nmol/L, and values were even lower when a low-polyphenol diet was given to the volunteers before a test meal (45, 46). The baseline concentration slightly increased (165 nmol/L) after 6-wk supplementation with 500 mg/d pure rutin (32). The increase was more pronounced in 2 other studies; plasma concentrations reached 1.5 μmol/L after 28 d of supplementation with a high dose of quercetin (>1 g/d) (47) and 0.63 μmol/L after supplementation with 80 mg/d quercetin equivalents for 1 wk (37). It should be noted that very high interindividual variability was observed in the latter study and in others (27, 34, 37). Some individuals could be better absorbers than others, possibly because of particular polymorphisms for intestinal enzymes or transporters. Quantitative data are still lacking for other flavonols and flavones.

FLAVANONES

Flavanones represent a small group of compounds, including glycosides of hesperetin present in oranges and glycosides of naringenin present in grapefruit. The bioavailability of the glycosides of eriodictyol, present in lemons, has never been studied in humans. The C_{max} values for flavanone metabolites were measured ~5 h after the ingestion of citrus fruits (**Table 3**). This is the time required for hydrolysis of the rhamnoglycosides hesperidin, naringin, and narirutin by the microflora, before absorption of the released aglycones in the colon. Aglycones are absorbed more rapidly; Bugianesi et al (50) showed that C_{max} was reached as early as 2 h after the ingestion of tomato paste, which

TABLE 3
Bioavailability studies of flavanones or flavanone-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
			h	*μmol/L*	*μmol · h/L*	*% of intake*	*h*	
Orange juice	5	110 or 220 mg eq hesperetin	5.4–5.8	0.46–1.28	4.19–9.28	4.1–6.4		48
Orange juice	5	22.6 or 45 mg eq naringenin	4.6–5	0.06–0.2	0.43–1.29	7.1–7.8		48
Orange juice	8	126 mg eq hesperetin	5.4	2.2	10.3	5.3	2.2	49
Orange juice	8	23 mg eq naringenin	5.5	0.64	2.6	1.1	1.3	49
Grapefruit juice	5	199 mg eq naringenin	4.8	5.99	27.7	30.2	2.2	49
Tomato paste	5	3.8 mg naringenin	2	0.12				50
Pure compound	6	500 mg pure naringin				4.0		51
Pure compound	1	500 mg pure naringin				4.8		52
Pure compound	1	500 mg pure hesperidin				3.0		52
Grapefruit juice	4	325 mg eq naringenin				6.8		52
Orange juice	4	44 mg eq hesperetin				24.4		52
Grapefruit juice	6	7.2 mg naringin/kg bw				8.9	2.6–2.9	53
Grapefruit juice	2	214 mg naringin/d for 1 wk				14d		54

[1] T_{max}, time to C_{max}; AUC, area under the curve; eq, equivalents; bw, body weight.

contains naringenin aglycone. However, natural foods rarely contain significant amounts of flavanones in the aglycone form.

Plasma metabolites of flavanones have not yet been identified. Monoglucuronides of hesperetin were shown to be the major forms present in plasma after ingestion of orange juice, but the positions of glucuronidation are still not known (48). Microbial metabolites such as *p*-hydroxyphenylpropionic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and phenylpropionic acid were produced with in vitro incubation of naringenin with human microflora (17, 55, 56). They were also detected in rat urine (57). The same types of microbial metabolites were detected for hesperetin (58, 59). Therefore, microbial metabolites may also be present in human plasma.

The total urinary excretion of conjugated flavanones accounted for 8.6% of the intake for hesperidin and 8.8% for naringin (Table 3). Plasma concentrations reached 1.3–2.2 μmol/L hesperetin metabolites with an intake of 130–220 mg given as orange juice (48, 49) and up to 6 μmol/L naringenin metabolites with 200 mg ingested as grapefruit juice (49). However, data are still scarce, with only 3 studies having investigated the bioavailability of flavanones in plasma.

CATECHINS

The daily intake of catechin and proanthocyanidin dimers and trimers has been estimated to be 18–50 mg/d, with the main sources being tea, chocolate, apples, pears, grapes, and red wine (60, 61). Although they are present in many fruits and in red wine, the bioavailability of catechins has been studied mainly after ingestion of cocoa or tea (**Table 4**).

Bioavailability differs markedly among catechins. By giving pure catechins individually, van Amelsvoort et al (78) demonstrated that galloylation of catechins reduces their absorption. They found that only epigallocatechin was methylated and that 4'-*O*-methyl-epigallocatechin accounted for 30–40% of the total metabolites of epigallocatechin. In another study, the 4'-*O*-methyl-epigallocatechin concentration was 5 times higher than that of epigallocatechin in plasma and 3 times higher than that in urine (84). Meng et al (74) recently showed that epigallocatechin gallate (EGCG) was also methylated into 4',4''-di-*O*-methyl-EGCG. The concentration of this metabolite was ~15% that of EGCG in human plasma. Catechin was also methylated but preferentially in the 3'-position (68). Only unchanged catechins were measured in most studies, whereas the methylated metabolites were not analyzed. Therefore, the mean bioavailability parameters calculated in this review for catechins are probably underestimated.

EGCG is the only known polyphenol present in plasma in large proportion (77–90%) in a free form (73–76). The other catechins are highly conjugated with glucuronic acid and/or sulfate groups. The exact nature of the major circulating metabolites of epicatechin has been elucidated, ie, epicatechin-3'-*O*-glucuronide, 4'-*O*-methyl-epicatechin-3'-*O*-glucuronide, 4'-*O*-methylepicatechin-5- or 7-*O*-glucuronide, and the aglycones epicatechin and 4'-*O*-methylepicatechin (89).

Microbial metabolites, namely, 5-(3',4',5'-trihydroxyphenyl) valerolactone, 5-(3',4'-dihydroxyphenyl)valerolactone, and 5-(3',5'-dihydroxyphenyl)valerolactone, mostly in conjugated forms, were also identified in plasma and urine of volunteers after ingestion of green tea (74). These metabolites accounted for 6–39% of the ingested epigallocatechin and epicatechin, 8–25 times the levels measured for the unchanged compounds (90). Because they appear later than catechins in plasma and have long half-lives, these compounds could prolong the actions of catechins (75). They probably exert some interesting antioxidant activity, because of their di-/trihydroxyphenyl groups.

Catechins are rapidly eliminated. Galloylated catechins were never recovered in urine (75, 76, 78). This is explained not by degalloylation, which has been shown to be a minor process in humans, but rather by preferential excretion of these compounds in bile (78). Extensive biliary excretion of EGCG was previously reported in rats (91).

PROANTHOCYANIDINS

Because of the lack of accurate data on the proanthocyanidin contents of foods, we are not yet able to provide a good estimation of the mean daily intake of these compounds. However, nearly one-half of 88 tested foods derived from plants were found to be dietary sources of proanthocyanidins, which suggests that these are among the most abundant polyphenols in our diet (92).

TABLE 4
Bioavailability studies of flavanols or flavanol-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
			h	*μmol/L*	*μmol · h/L*	*% of intake*	*h*	
Cocoa beverage	5	323 mg catechins	2	5.9 EC + 0.16 catechins				62
Chocolate (80 g)	10	137 mg EC	2	0.26				63
Cocoa	6	1.53 mg/kg bw	2	1–1.5			1.7–3	64
Cocoa	5	220 mg EC	2	4.92		25.3		65
Chocolate	5	220 mg EC	2	4.77		29.8		65
Chocolate	20	46, 92, 138 mg EC	2	0.13, 0.26, 0.36				66
Chocolate (40, 80 g)	8	82, 164 mg EC	2–2.6	0.38, 0.7	1.53, 3.7		1.9–2.3	67
Red wine (120 mL)	9	35 mg catechin	1.5	0.091	0.36		3.1	68
Red wine (120 mL)	9	35 mg catechin	1.44	0.077	0.31		3.2	69
Red wine (120 mL)	9	35 mg catechin				3–10		70
Pure catechin	12	0.36 mg/kg bw	0.5	0.14–0.49		1.2–3		36
Pure catechin	3	2 g	2–3	2.8–5.9	22–37	55.0		71
Pure catechin	6	0.5, 1, 2 g	1.4–2	2, 3.8, 7.8	4.5, 9.7, 20.1	23.6–28.2	1–1.3	72
Pure EGCG	6 × 8	50, 100, 200, 400, 800, 1600 mg	1.3–2.2	0.28, 0.39, 0.72, 1.36, 2.33, 7.4 EGCG	0.9, 2.6, 2.7, 5.5, 8.3, 22.4		1.9–4.6	73
Pure EGCG	4	2 mg/kg bw	2	0.097 EGCG + 0.018 4', 4″diMe EGCG	0.52 EGCG + 0.1 diMe EGCG	0.1	2.5 EGCG, 2.8 4', 4″diMe EGCG	74
Pure EGCG	8	2 mg/kg bw	1.6	0.075 EGCG	0.47		3.7	75
Pure EGCG	4 × 5	200, 400, 600, 800 mg	1.8–4	0.16, 0.24, 0.37, 0.96 EGCG	0.8, 1.3, 3.7, 6.1		1.9–3.1	76
Polyphenon E	4 × 5	200, 400, 600, 800 mg	2.4–4.1	0.16, 0.27, 0.36, 0.82 EGCG	0.8, 1.9, 2.9, 5.9		1.9–3	76
Green tea powder	4	105 mg EGCG	2	0.14–0.31 EGCG				77
Pure EGCG	10	688 mg	2.9	1.3 EGCG	12.1	<0.02	3.9	78
Pure EGCG	10	459 mg	1.7	5 EGC + 1.9 Me EGC	20.1 EGC + 12.6 Me EGC	9.8 EC + 3.8 Me EGC	1.7 EGC, 2.5 Me EGC	
Pure EC gallate	10	663 mg	4	3.1 EC gallate	39.9	<0.02	6.9	
Green tea extract	3	225, 375, 525 mg EGCG		0.66, 4.3, 4.4 EGCG at 1.5 h				79
		7.5, 12.5, 17.5 mg EGC		0.03, 0.14, 0.25 EGC at 1.5 h				
Green tea extracts	8	2.8 mg EGCG/kg bw	1.6	0.17 EGCG	1.11	Trace amount	3.4 EGCG	80
		2.2 mg EGC/kg bw	1.3	0.73 EGC + 5.05 Me EGC	3.09	3.3 EGC + 12.3 4'-Me EGC	1.7 EGC	
		0.64 mg EC/kg bw	1.3	0.43 EC	1.82	8.9 EC	2.0 EC	
Green tea extract	4	88 mg EGCG		0.24 EGCG at 1 h				81
		82 mg EGC		0.46 EGC at 1 h		2.0 total catechins		
		32 mg EC		0.21 EC at 1 h				
Green tea extract	6	109.5, 219, 328 mg EGCG	1.6; 2.4; 2.7	0.26, 0.71, 0.70 EGCG	1.96, 4.85, 5.37		5.5, 5.0, 4.9	82
		102, 204, 306 mg EGC	1.4; 1.8; 1.3	0.48, 1.66, 1.8 EGC	2.02, 8.14, 10.72		2.7, 2.8, 2.5	
		37.5, 75, 112.5 mg EC	1.4; 1.8; 1.8	0.19, 0.65, 0.65 EC	0.96, 3.46, 4.13		5.7, 3.4, 3.2	
Polyphenon	5	164 mg total catechins		0.56 total catechins at 3 h				83
		100 mg EGCG		0.26 EGCG at 3 h				
Green tea extracts	12	0.93 g total catechins	2.3	0.55 total catechins	2.22		4.8	84
Green tea extracts	4	1.64 mg EGC/kg bw	0.5–2	0.8–1.2 EGC + 3.8–6.9 4' MeEGC			1.0 EGC; 4.4 4' Me EGC	85
Green tea	21	640 mg total catechins	1.5	1.8 total catechins				86

Continues

TABLE 4
Continued

Source	No. of subjects	Dose	T_{max}	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
Green tea	18	1.04 g total catechins/d for 3 d	0.5–2	1.0 total catechins		4.2 EGC, 6.5 EC		87
Black tea	12	0.3 g total catechins	2.2	0.17 total catechins	0.53		6.9	83
Black tea + milk	12	0.3 g total catechins	2	0.18 total catechins	0.60		8.6	83
Black tea	15	400 mg total catechins/4 times		0.02 EGCG, 0.14 EGC		0.14 EGCG, 3.7 EGC		88
Black tea	21	140 mg total catechins	1.5	0.34 total catechins				85
Black tea	18	400 mg total catechins/d for 3 d		0.3 total catechins		2.5 EGC, 6.5 EC		86

[1] T_{max}, time to C_{max}; AUC, area under the curve; bw, body weight; EC, epicatechin; EGC, epigallo catechin; Me, methyl.

Polymeric proanthocyanidins are not absorbed as such. The detection of proanthocyanidin dimers B1 and B2 in human plasma was reported in only 2 studies (62, 93) (**Table 5**). The absorption of these dimers was minor, ~100-fold lower than that of the flavanol monomers in the study by Holt et al (62). In vitro and animal studies confirmed that polymerization greatly impairs intestinal absorption (94–96).

However, health effects of proanthocyanidins may not require efficient absorption through the gut. Indeed, these compounds may have direct effects on the intestinal mucosa and protect it against oxidative stress or the actions of carcinogens. In addition, the consumption of proanthocyanidin-rich foods, such as cocoa, red wine, or grape seed extracts, has been shown to increase the plasma antioxidant capacity, to have positive effects on vascular function, and to reduce platelet activity in humans (97). These procyanidin-rich sources always contain 5–25% monomers or other polyphenols, which leaves doubts about whether proanthocyanidins are actually the active compounds in these sources. If they are, then they may have effects through interactions with other components, such as lipids or iron, in the gut.

Biological effects may be attributable not to direct actions of proanthocyanidins themselves but to actions of some of their metabolites that can be more readily absorbed. On the basis of in vitro experiments, Spencer et al (98) suggested that polymers could be degraded into monomers during their transit in the stomach. However, Rios et al (99) clearly demonstrated that this does not occur in humans, probably because the food bolus has a buffering effect, making the acidic conditions milder than required for proanthocyanidin hydrolysis.

Proanthocyanidins are degraded into various aromatic acids by the microflora. The incubation of purified, ^{14}C-labeled, proanthocyanidin oligomers with human colonic microflora led to the formation of *m*-hydroxyphenylpropionic acid, *m*-hydroxyphenylacetic acid, and their *p*-hydroxy isomers, *m*-hydroxyphenylvaleric acid, phenylpropionic acid, phenylacetic acid, and benzoic acid (100). Some of these compounds, namely, *m*-hydroxyphenylpropionic acid and *m*-hydroxyphenylacetic acid, as well as *m*-hydroxybenzoic acid, were shown to increase in human urine after consumption of procyanidin-rich chocolate (101). However, the microbial metabolism of proanthocyanidins has never been studied in humans after consumption of purified proanthocyanidin polymers. By feeding rats with purified catechin, dimer B3, trimer C2, or procyanidin polymers, Gonthier et al (102) showed that the extent of degradation into aromatic acids decreased as the degree of polymerization increased; it was 21 times lower for polymers than for the catechin monomer, probably because of the antimicrobial properties and protein-binding capacity frequently described for proanthocyanidins. Therefore, the quantitative importance of the degradation of proanthocyanidins into microbial metabolites must be further evaluated in humans.

ISOFLAVONES

Isoflavones are provided only by soybean-derived products. They can be present as aglycones or glycosides, depending on the soy preparation. Some authors investigated the differences in bioavailability between aglycones and glycosides by using pure molecules. Contradictory results have been obtained (**Table 6**). Setchell et al (112) found greater bioavailability of glucosides, as measured from the areas under the plasma concentration-time curves. Izumi et al (110) found greater bioavailability of aglycones, on the basis of C_{max}, but they did not measure isoflavone concentrations between 6 and 24 h, whereas Setchell et al (112) reported that the mean time to reach C_{max} was prolonged to 9 h after glycoside ingestion. Two other studies found no significant differences in the absorption efficiency for aglycones and glycosides (117, 118).

In contrast, equol production was significantly higher after ingestion of daidzin than after ingestion of daidzein (112, 117). Equol is a bacterial metabolite that has been shown to be more

TABLE 5
Bioavailability studies of proanthocyanidins or proanthocyanidin-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	Ref
			h	*μmol/L*	
Cocoa beverage	5	256 mg dimers	2	0.041 B2	62
Grapeseed extract	4	18 mg procyanidin B1		0.011 B1	93

[1] T_{max}, time to C_{max}.

TABLE 6
Bioavailability studies of isoflavones or isoflavone-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
			h	*μmol/L*	*μμnol · h/L*	*% of intake*	*h*	
Soybean milk	12	24.7, 45.9, 70.7 mg Da		0.79, 1.22, 2.24 at 6.5 h		19.8, 23.7, 20.8		103
		19.3, 36.2, 55.7 mg Ge		0.53, 1.10, 2.15 at 6.5 h		5.3, 11.0, 10.0		
Tofu or texturized vegetable proteins	7	0.34–0.41 mg Da/kg bw 0.48–0.56 mg Ge/kg bw		1.44 at 6.5 h 1.33 at 6.5 h		49.0 13–16		104
Soybean flour in cow milk	6	0.67 mg Da/kg bw 0.97 mg Ge/kg bw	7.4 8.4	3.14 4.09		62.0 22.0	4.7 5.7	105
Baked soybean powder	7	26.1 mg Da 30.2 mg Ge	8.0 8.0	1.56 2.44		35.8 Da + 7 equal 17.6 Ge	5.8 8.4	106
Soymilk	14	0.49 mg Da/kg bw 0.59 mg Ge/kg bw 0.10 mg Gly/kg bw		1.14 at 6 h 1.74 at 6 h 0.21 at 6 h		48.6 27.8 55.3		107
Soygerm	14	0.55 mg Da/kg bw 0.15 mg Ge/kg bw 0.50 mg Gly/kg bw		1.40 at 6 h 0.49 at 6 h 0.79 at 6 h		43.8 29.7 54.5		107
Cooked soybeans	10	20 mg Da 24 mg Ge				45.0 13.0		108
Texturized vegetable protein	10	28 mg Da 32 mg Ge				51.0 13.0		
Tofu	5	37 mg Da 43 mg Ge				50.0 16.0		
Tempeh	4	22 mg Da 30 mg Ge				38.0 9.0		
Soy beverage	12	0.6 mg Da/kg bw 1 mg Ge/kg bw 0.1 mg Gly/kg bw				26.8 6.81 19.0		109
Soybean extracts	8	15.7, to 233.7 mg Da 13, 210.6 mg Ge 20.8, 333.1 mg daidzin 25.9, 388.8 mg genistin	2, 4 2, 4 4, 4 4, 6	0.77, 16.6 1.04, 21.2 0.17, 3.66 0.35, 2.56				110
Soy beverage	12	0.6 mg Da/kg bw 1 mg Ge/kg bw	5.5 4.4	0.3 0.65	2.3 8.7		3.4 7.9	111
Pure compounds	6 4 6 3 1	50 mg Da 50 mg daidzin 50 mg Ge 50 mg genistin 25 mg Gly	6.6 9 5.2 9.3 4–6	0.76 1.55 1.26 1.22 0.72	11.6 17.8 16.8 18.3 2.5		9.3 4.6 6.8 7.0 8.9	112
Soy isolates	30	0.5–7.8mg Da/kg bw 1–16 mg Ge/kg bw		0.6–16.9 0.9–27		14–53 Da 4–18 Ge		113
Soy extract	24	0.28–8.4 mg Da/kg bw 2, 4, 8, 16 mg Ge/kg bw	2.5–11 3–9.5	1.7–9.0 3.4–25.4	14.1–134.8 35.4–337.9	26–42 9.5–14		114
Soy nuts	10	6.6, 13.2, 26.4 mg Da 9.8, 19.6, 39.2 mg Ge	5.8, 6.4, 6.0 4.9, 4.0, 6.0	0.4, 0.84, 1.65 0.59, 1.22, 2.21	5.72, 10.1, 18.1 10.1, 17.3, 31.2	63, 54, 44 25.2, 13.4, 15.8	8.7, 7.9, 7.5 10.8, 10.0, 9.6	115
^{13}C-labeled compounds	16	0.4, 0.8 mg Da/kg bw 0.4, 0.8 mg Ge/kg bw		0.31, 0.71 0.55, 0.87	4.0, 8.7 6.7, 9.8	29.5, 25.6 8.9, 8.3	8.2, 7.2 7.5, 7.4	116
Pure aglycones	15	16 mg Da 13.8 mg Ge	5.0 4.2	0.53 0.53	6.2 Da + 7 equol 8.9			117
Pure glycosides		12.5 mg Da eq 17.2 mg Ge eq	4.0 5.3	0.40 0.57	8.3 Da + 9 equol 8.3			

[1] T_{max}, time to C_{max}; AUC, area under the curve; bw, body weight; Da, daidzein; Ge, genistein; Gly, glycitein; eq, equivalents.

estrogenic than its precursor daidzein in many in vitro studies and in animal models (119). There is great interindividual variability in the capacity to produce equol, and only 30-40% of the Western population are "equol producers." Equol producers may gain more benefits from soy consumption than do nonproducers (119, 120). Therefore, it would be interesting to find a way to make nonproducers become producers. To date, no clear correlations between dietary habits or microflora composition and the capacity to produce equol have been reported. It would be interesting to separate volunteers into equol producers and nonproducers in future intervention studies designed to investigate the effects of soy isoflavones. C_{max} values for equol were measured 12–24 h after isoflavone ingestion (112, 117).

It has long been thought that the greater urinary excretion of daidzein reflects greater bioavailability of this isoflavone, compared with genistein (103). The explanation is that a greater fraction of genistein is eliminated in bile, as observed in rats (121). Plasma kinetic curves often showed a first peak followed

TABLE 7
Bioavailability studies of hydroxycinnamic acids or hydroxycinnamic acid-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	Urinary excretion	Ref
			h	*nmol/L*	*% of intake*	
Coffee (200 mL)	10	96 mg chlorogenic acid	1	505 caffeic acid		135
Red wine (100, 200, 300 mL)	5	0.9–1.8–2.7 mg caffeic acid	1	6.6–18–27		136
Red wine (200 mL)	10	1.8 mg caffeic acid	0.5–1	37–60		137
Pure compound	7 ileostomized	1 g chlorogenic acid			0.3	138
Pure compound	7 ileostomized	500 mg caffeic acid			10.7	138
Coffee	5	898 mg eq chlorogenic acid/3 times			5.9[2]	139
Artichoke extract	10	124 mg eq chlorogenic acid/3 times		12–43 ferulic acid	5.6[2]	140
Red wine	12	55 μg caffeic acid/kg bw	2	84		141
Apple cider (1.1 L)	6	15 mg total hydroxycinnamic acids	<2	430		35
Breakfast cereals	6	260 mg ferulic acid	1–3	150–210 ferulic acid	3.1	142
Tomatoes	5	30 mg ferulic acid			11–25	143
Beer (4 L)	5	9.4 mg ferulic acid			61.7	144

[1] T_{max}, time to C_{max}; eq, equivalents; bw, body weight.
[2] Ferulic + isoferulic + dihydroferulic + vanillic acids.

~3 h later by a second peak, reflecting enterohepatic cycling (112, 117). By using ^{13}C-labeled daidzein and genistein, Setchell et al (116) recently showed that the systemic bioavailability and C_{max} were significantly higher for genistein than for daidzein. The limited data for glycitein indicate greater bioavailability than for the other isoflavones (107, 114).

The nature of isoflavone metabolites was the same after glycoside or aglycone ingestion. Glycosides are hydrolyzed before absorption and are not recovered as such in plasma (122). Aglycones have been recovered in small proportions, generally <5% of the total metabolites (111–113, 123). The main metabolites are 7-*O*-glucuronides and 4'-*O*-glucuronides, with small proportions of sulfate esters (111, 123, 124). Additional metabolites have been identified in human plasma or urine, including dihydrodaidzein, dihydrogenistein, dihydroequol, *O*-desmethylangolensin, and 6-hydroxy-*O*-desmethylangolensin (125–127).

Elimination of isoflavones is quite slow, with half-life values of 6–8 h (Table 6). After ingestion of daidzein or genistein at 0.4 or 0.8 mg/kg body weight, baseline concentrations of isoflavones in plasma were regained only after ~48 h (116). Plasma concentrations should therefore increase with repeated ingestion of soy products. However, Lu et al (128) reported that relative urinary excretion of isoflavones and elimination half-lives progressively decreased during 4 wk of daily soymilk ingestion. Lampe et al (129) did not observe any effect on urinary excretion of 1-mo supplementation with isoflavones.

Another point worth noting is the evidence that high concentrations of isoflavones can be found in breast tissue of premenopausal women and in prostate glands of men (130–132). These are the only available data on polyphenol concentrations in tissues.

HYDROXYCINNAMIC ACIDS

Intake of chlorogenic acid varies widely but may be very high, up to 800 mg/d among coffee drinkers (133, 134). Nevertheless, very few studies have addressed the bioavailability of this hydroxycinnamic acid, in comparison with other polyphenols (**Table 7**).

Olthof et al (138) showed that the esterification of caffeic acid, as in chlorogenic acid, markedly reduced its absorption. This was also observed in rats (145, 146). In fact, the absorption of chlorogenic acid occurs mainly in the colon, after hydrolysis by microbial esterases. It is not clear whether chlorogenic acid is present, as such or in a conjugated form, in human plasma. Nardini et al (135) found only caffeic acid in plasma after the ingestion of coffee. We observed, however, that the preparation of β-glucuronidase from *Helix pomatia* that is generally used to hydrolyze samples also contains esterases that are able to degrade chlorogenic acid into caffeic acid. Therefore, the possibility that chlorogenic acid is present in plasma but is hydrolyzed during sample treatment cannot be excluded. Intact chlorogenic acid has been detected at low concentrations in nonhydrolyzed urine samples (138, 147). Metabolites other than caffeic acid have been identified after ingestion of chlorogenic or caffeic acid, namely, ferulic acid, isoferulic acid, dihydroferulic acid, vanillic acid, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyhippuric acid, and hippuric acid (139, 140, 147). Their quantitative importance remains to be investigated.

Ferulic acid is another abundant hydroxycinnamic acid. When present in free form in tomatoes or beer, it is efficiently absorbed (143, 144). However, ferulic acid is also the main polyphenol present in cereals, in which it is esterified to the arabinoxylans of the grain cell walls. This binding has been reported to hamper the absorption of ferulic acid in rats (148, 149). In humans, Kern et al (142) measured the urinary excretion and plasma concentrations of ferulic acid metabolites after ingestion of breakfast cereals. They deduced from the kinetic data that absorption of ferulic acid from cereals takes place mainly in the small intestine, from the soluble fraction present in cereals. Only a minor amount of ferulic acid linked to arabinoxylans was absorbed after hydrolysis in the large intestine.

HYDROXYBENZOIC ACIDS

Very little is known about the absorption and metabolism of hydroxybenzoic acids (150). Their limited distribution in food has resulted in limited interest by nutritionists. However, the few studies addressing the bioavailability of gallic acid in humans revealed that this compound is extremely well absorbed, compared with other polyphenols (**Table 8**). Plasma concentrations of free and glucuronidated forms of gallic acid and its main

TABLE 8
Bioavailability studies of gallic acid or gallic acid-containing foods[1]

Source	No. of subjects	Dose	T_{max}	Plasma concentration	AUC	Urinary excretion	Elimination half-life	Ref
			h	*μmol/L*	*μmol h/L*	*% of intake*	*h*	
Pure compound	1	50 mg GA		1.8 GA + 2.3 4-MeGA		37.1		151
Pure compound	10	50 mg GA	1.3–1.5	1.8 GA + 2.8 4-MeGA	4.3 GA + 9.6 MeGA	36.4	1.2–1.5	152
Assam black tea	10	50 mg GA	1.4–1.5	2.1 GA + 2.6 4-MeGA	4.5 GA + 9.0 MeGA	39.6	1.1–1.3	152
Red wine (300 mL)	2	4 mg GA		0.22 GA + 1.1 4-MeGA + 0.25 3-MeGA				153
Red wine	12	47μg GA/kg bw	2	0.18 4-MeGA				141

[1] T_{max}, time to C_{max}; AUC, area under the curve; GA, gallic acid; MeGA, methylgallic acid.

metabolite 4-*O*-methylgallic acid reached 4 μmol/L after ingestion of 50 mg pure gallic acid. Such intake is not inconceivable, because red wine usually contains 10–60 mg/L gallic acid. However, gallic acid exists in different forms in fruits, nuts, tea, and red wine, ie, the free form, esterified to glucose (as in hydrolyzable tannins), or esterified to catechins or proanthocyanidins (92, 154). It would be interesting to compare the bioavailability of the different forms of gallic acid.

COMPARATIVE BIOAVAILABILITY OF POLYPHENOLS

Mean values for C_{max}, time to reach C_{max}, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion (related to the ingested dose) were calculated for the different polyphenols (**Table 9**), on the basis of the data compiled in Tables 1–8. Only data from studies using a single dose of a well-characterized polyphenol source were taken into account. To facilitate comparisons between polyphenols, data were converted to correspond to the same supply of polyphenols, a single 50-mg dose of aglycone equivalent. For this, we assumed that the bioavailability parameters increase linearly with the dose, which has been demonstrated in humans only for EGCG (73). When several doses were investigated in the same study, only a mean value for the whole study was considered.

The most striking result of this survey was that gallic acid is far better absorbed than the other polyphenols. The C_{max} values for its metabolites reached 4 μmol/L with a 50-mg intake, and the relative urinary excretion was 38%. Next are isoflavones, which are the most well-absorbed flavonoids, with C_{max} values of ~2 μmol/L after a 50-mg intake and mean relative urinary excretions of 42% for daidzin and 15.6% for genistin. Proanthocyanidins and anthocyanins are very poorly absorbed but, in the case of anthocyanins, all of the metabolites might not have been identified, resulting in underestimation of their bioavailability. Values for catechins are certainly underestimated, because methylated metabolites were not taken into account in some studies. Data are

TABLE 9
Compilation of pharmacokinetic data from 97 bioavailability studies[1]

	T_{max}		C_{max}		AUC		Urinary excretion		Elimination half-life	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	h		*μmol/L*		*μmol h/L*		*% of intake*		*h*	
Daidzin	6.3 ± 0.6	4.0–9.0	1.92 ± 0.25	0.36–3.14	21.4 ± 6.5	2.7–38.6	42.3 ± 3.0	21.4–62.0	5.3 ± 0.8	3.4–8.0
Daidzein	4.9 ± 1.0	3.0–6.6	1.57 ± 0.52	0.76–3.00	12.2 ± 2.9	7.5–17.4	27.5		8.5 ± 0.8	7.7–9.3
Genistin	6.5 ± 0.6	4.4–9.3	1.84 ± 0.27	0.46–4.04	23.7 ± 6.7	6.2–45.1	15.6 ± 1.8	6.8–29.7	7.8 ± 0.7	5.7–10.1
Genistein	4.1 ± 0.6	3.0–5.2	2.56 ± 1.00	1.26–4.50	19.8 ± 6.5	10.4–32.2	8.6		7.1 ± 0.3	6.8–7.5
Glycitin	5.0		1.88 ± 0.38	1.50–2.26	7.9		42.9 ± 12.0	19.0–55.3	8.9	
Hesperidin	5.5 ± 0.1	5.4–5.8	0.46 ± 0.21	0.21–0.87	2.7 ± 0.7	1.9–4.1	8.6 ± 4.0	3–24.4	2.2	
Naringin	5.0 ± 0.2	4.6–5.5	0.50 ± 0.33	0.13–1.50	3.7 ± 1.5	0.9–7.0	8.8 ± 3.17	1.1–30.2	2.1 ± 0.4	1.3–2.7
Quercetin glucosides	1.1 ± 0.3	0.5–2.9	1.46 ± 0.45	0.51–3.80	9.8 ± 1.9	5.7–16.0	2.5 ± 1.2	0.31–6.4	17.9 ± 2.2	10.9–28.0
Rutin	6.5 ± 0.7	4.3–9.3	0.20 ± 0.06	0.09–0.52	2.9 ± 0.9	1.6–5.5	0.7 ± 0.3	0.07–1.0	19.9 ± 8.1	11.8–28.1
(Epi)catechin	1.8 ± 0.1	0.5–2.5	0.40 ± 0.09	0.09–1.10	1.1 ± 0.3	0.5–2.0	18.5 ± 5.7	2.1–55.0	2.5 ± 0.4	1.1–4.1
EGC	1.4 ± 0.1	0.5–2.0	1.10 ± 0.40	0.30–2.70	2.0 ± 0.8	1.0–3.6	11.1 ± 3.5	4.2–15.6	2.3 ± 0.2	1.7–2.8
EGCG	2.3 ± 0.2	1.6–3.2	0.12 ± 0.03	0.03–0.38	0.5 ± 0.1	0.2–0.9	0.06 ± 0.03	0.0–0.1	3.5 ± 0.3	2.5–5.1
Gallic acid	1.6 ± 0.2	1.3–1.5	4.00 ± 0.57	2.57–4.70			37.7 ± 1.0	36.4–39.6	1.3 ± 0.1	1.1–1.5
Chlorogenic acid	1.0		0.26				0.3			
Caffeic acid	1.4 ± 0.6	0.7–2.0	0.96 ± 0.26	0.45–1.35			10.7			
Ferulic acid	2.0		0.03				27.6 ± 17.6	3.1–61.7		
Anthocyanins	1.5 ± 0.4	0.7–4.0	0.03 ± 0.02	0.001–0.20			0.4 ± 0.3	0.004–5.1		
Proanthocyanidin dimers	2.0		0.02 ± 0.01	0.008–0.03						

[1] All data were converted to correspond to a supply of 50 mg aglycone equivalent.
T_{max}, time to reach C_{max} AUC, area under the plasma concentration-time curve EGC, epigallocatechin.

still scarce for hydroxycinnamic acids, and the calculated mean values are probably not very reliable.

The mean area under the plasma concentration-time curve, C_{max}, and urinary excretion values clearly show the lower absorption of rutin, compared with quercetin glucosides. Another observation is that galloylation of epigallocatechin markedly reduces its absorption. Gallic acid, quercetin glucosides, catechins, free hydroxycinnamic acids, and anthocyanins, which are absorbed in the small intestine or the stomach, reached C_{max} at ~1.5 h, whereas rutin and the flavanones hesperidin and naringin, which are absorbed after release of the aglycones by the microflora, reached C_{max} at ~5.5 h. The mean time to reach C_{max} for chlorogenic acid is surprising, because this compound also must be hydrolyzed by the microflora before absorption. In the sole study considered, however, chlorogenic acid was provided as a liquid (coffee) to fasted volunteers, which might have accelerated the absorption kinetics.

Relative urinary excretion is currently used to estimate the minimal absorption rate but, when polyphenols are highly excreted in bile, as for EGCG and genistein, absorption is underestimated. For most polyphenols, the urinary excretion values were consistent with the plasma kinetic data. Values ranged from 0.3% to 43% of the intake, which demonstrates the great variability in the bioavailability of the different polyphenols.

With respect to the elimination half-lives, it appears that catechins, gallic acid, and flavanones have no chance to accumulate in plasma with repeated ingestion. Some of their metabolites may have longer half-lives, however, and quercetin, with a longer half-life, could accumulate in plasma with repeated ingestion.

Extensive variability was observed among the studies. Tenfold variations in the C_{max} values were observed for most compounds. Several factors may explain the variability, such as the food matrix or background diet. Interindividual variations are also important, and some people might have different levels of metabolizing enzymes or transporters, enabling more efficient absorption of polyphenols.

It is important to realize that the mode of calculation and representation used in this review does not take into account the mean dietary intake of each polyphenol. For example, even if isoflavones are efficiently absorbed, they are usually not the major circulating polyphenols in Western populations, because the isoflavone intake is far lower than 50 mg/d for these populations. In contrast, a single glass of orange juice easily provides 50 mg hesperidin.

CONCLUSIONS

Bioavailability varies widely among polyphenols and, for some of compounds, among dietary sources, depending on the forms they contain. The plasma concentrations of total metabolites range from 0 to 4 μmol/L with an intake of 50 mg aglycone equivalents. The polyphenols that are most well absorbed in humans are isoflavones and gallic acid, followed by catechins, flavanones, and quercetin glucosides, with different kinetics. The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins. Data for other polyphenols are still too limited. The plasma kinetics also differ among polyphenol classes, with C_{max} being reached after ~1.5 h or ~5.5 h, depending on the site of intestinal absorption. This information should be useful for the design and interpretation of intervention studies investigating the health effects of polyphenols.

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